PHARMACOGNOSTICAL, PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF THE LEAVES OF *Cordia obliqua Willd.* (Boraginaceae)

Dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University,

Chennai

In partial fulfillment of the requirements

For the award of the Degree of

MASTER OF PHARMACY
IN
PHARMACOGNOSY

SUBMITTED
BY
261220705

DEPARTMENT OF PHARMACOGNOSY
MADURAI MEDICAL COLLEGE
MADURAI – 625020

APRIL-2014

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Dated

CERTIFICATE

This is to certify that the dissertation entitled **"PHARMACOGNOSTICAL, PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF THE LEAVES OF *Cordia obliqua willd.* (Boraginaceae)''** submitted by **Miss.M.KALAIYARASI (Reg. No.261220705)** in partial fulfillment of the requirements for the award of the degree of **MASTER OF PHARMACY** in **PHARMACOGNOSY** by The Tamil Nadu Dr. M.G.R. Medical University is a bonafied work done by her during the academic year 2013-2014 at the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai-625 020.

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Assistant Reader,
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Miss.R.GOWRI

(Project supervisor)

- **Part B**

Protocol form for research proposals to be submitted to the committee / institutional animal ethics committee, for new experiments or extensions of ongoing experiments using animals other than non – human primates

Project title: **ANTI-INFLAMMATORY ACTIVITY OF ETHANOLIC EXTRACT OF CORDIA OBLIQUA AGAINST CARRAGEENAN INDUCED PAW EDEMA IN RATS**

- Chief Investigator
  a. Name : Mr. N. Chidambaranathan, M.Pharm, Ph.D.,

  b. Designation : Head, Department of Pharmacology,

  K. M. College of Pharmacy,

  Uthangudi, Melur road,

  Madurai – 625 107

  c. Telephone Number : 0452 – 2424652, 2423452

- List of names of all individuals authorized to conduct procedures under this proposal.
  A. Mrs. G. Nalini, M.Pharm., (Pharmacological Supervisor).

  B. Mr. N.Jegan.M.Pharm, (Pharmacological Supervisor).

- Funding source: Nil
- Duration of the project
  a. Number of months : Five months

  b. Date of initiation : 01.10.2013.

  c. Date of completion : 28-02-2014.

- If date by which approval is needed is less than six weeks from date of submission, justification for the same.
  NIL

- A] To evaluate the anti-inflammatory activity of **Ethanolic extract of *Cordial obliquaa*** willd.
- Animals required:
  - Species: Albino Wistar rats.
  - Age/weight/size: 6 months, Rat (180-220gms)
    Medium.
  - Gender: Male and Female in equal ratios.
  - Numbers to be used: Total Numbers:24
  - Number of days each animal will be housed:30 Days.
- Rationale for animal usage:

(For IAEC / CPCSEA usage)

Proposal number	: M.Kalaiarasi/M.Pharm/IAEC/120 /KMCP/ 261220705/2013-14
Date first received	:01.10.2013
Date received after modification (if any)	: NA
Date received after second modification (if any)	: NA
Approval date	: 10.10.2013
Expiry date	: 28.02.2014
Name of IAEC / CPCSEA chairperson	: N.CHIDAMBARANATHAN

Date:10.10.2013

CPCSEA NOMINEE
INSTITUTIONAL ANIMAL ETHICS COMMITTEE
K.M. COLLEGE OF PHARMACY
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Signature

I. A. E. C. CHAIRMAN
INSTITUTIONAL ANIMAL ETHICAL COMMITTEE
K. M. COLLEGE OF PHARMACY
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ANNEXURE

Investigator declaration

- I certify that I have determined that the research proposal herein is not unnecessarily duplicate of previously reported research.
- I certify that all individuals working on this proposal and experimenting on the animals have been trained in animal handling procedures.
- For procedures listed under item 11, I certify that I have reviewed the pertinent scientific literature and have found no valid alternative to any procedure described herein which may cause less pain or distress.
- I will obtain approval from the IAEC / CPCSEA before initiating any significant changes in this study.
- Certified that performance of experiment will be initiated only up on review and approval of scientific intent by appropriate expert body (institutional scientific advisory committee / funding agency / other body (to be named)
- Institutional biosafety committee (IBC) certification of review and concurrence will be taken (required for studies utilizing DNA agents of human pathogens)
- I shall maintain all the records as per format (Form D)

M. Kalaiyalasi

Signature

(M.Kalaiarasi)

Name of Investigator

I. A. E. C. CHAIRMAN
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Dr. (Mr.) D. STEPHEN., M.Sc., Ph.D.,

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DEPARTMENT OF BOTANY,

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CERTIFICATE

This is to certify that the specimen brought by **Miss.M.Kalaiyarasi** ,II.M. Pharmacy, Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai is identified as ***Cordia obliqua willd.*** belonging to the family **Boraginaceae.**

SSTATION : Madurai.

(Dr. D. STEPHEN)

DATE :

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M.KALAIYARASI

CONTENTS

INTRODUCTION

CHAPTER - I

INTRODUCTION

Traditional medicine is the sum total of the knowledge, skills and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or nor used in the maintenance of health as well as in the prevention, diagnosis, or treatment of physical and mental illness.

Traditional medicine that has been adopted by other populations (outside its indigenous culture) is often termed alternative or complementary medicine.

Herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products that contain parts of plants or other plant materials as active ingredients [(1).]

In the early stages, the science of medicine developed around those plants which had curative properties. A continued search for medicinal plants during the last several centuries has given rise to a long list of plants which are of great use in the treatment of diseases and for promoting health. It can be stated, more or less truthfully, that every disease has a cure with a plant growing in nature.

The main limitation is the lack of standardization of raw materials, of processing methods and of the final products, dosage formulation and the non- existence of criteria for quality control. Research has to be directed to the use of modern scientific methodology and techniques to standardize all these different steps and quality control [(2).]

Herbal medicine

The world health organization(WHO) has recently defined traditional medicine (including herbal drugs) as comprising therapeutic practices that have been in existence, often for hundreds of years, before the development and spread of modern medicine and are still in

use today. The traditional medicine is the synthesis of therapeutic experience of generations of practising physicians of indigenous system of medicine [3].

The role of herbal medicines in traditional healing

The pharmacological treatment of diseases began long ago with the use of herbs. Methods of folk healing throughout the world commonly use herbs as part of their tradition. Some of these traditions are providing some examples of the array of important healing practices around the world that use herbs for this purpose [4].

Natural products for modern medicine

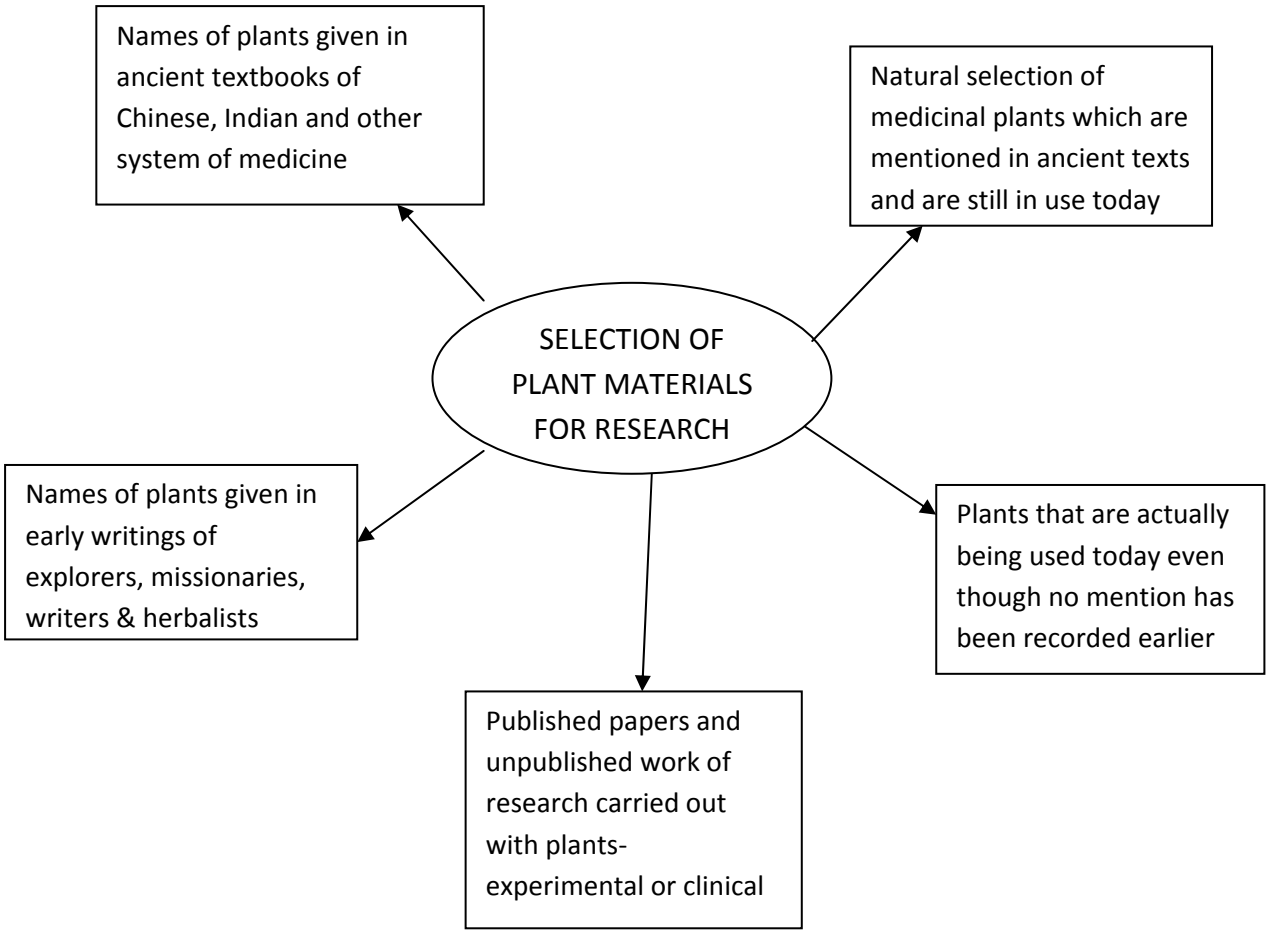
Plants are being used in medicine from time immemorial because they have fitted the immediate personal need, they are accessible and inexpensive, the practitioners speak to those who have used them in their own language and they are not provided from a remote professional or Government apparatus. For these and other reasons, the use of plants for medicines around the world still vastly exceeds the use of modern synthetic drugs. Such activity is not completely dismissed in scientific society and plants are also appreciated in pharmaceutical research as the major resource for new medicines and a growing body of medical literature supports the clinical efficacy of herbal treatments. Even where traditional use has largely died out in developed countries, there is an increasing yearning for a new deal in healthcare in which the old remedies feature strongly.

Researchers have no doubt that nature is still the preeminent synthetic chemist and that in plants particularly, there are almost infinite reserves of fascinating chemical constituents with actual and potential effects on the human body. As such information accumulates, it becomes possible to better understand traditional uses of plants.

Natural products will continue to be important in three areas of drug discovery

- As targets for production by biotechnology
- As a source of new lead compounds of novel chemical structure and

- As the active ingredients of useful treatments derived from traditional systems of medicine.



Safaty in herbal drugs

Major differences in the assessment of quality, safety and efficacy would hinder free circulation of herbal medicinal products may represent a risk for consumers. The complexity of herbal drug preparations and the interpretation of bibliographic data on safety and efficacy reflecting the experience gathered during long- term use are best addressed by involving specific expertise and experience. Safety and efficacy of complex biological products, such as herbal medicinal products are directly linked to pharmaceutical details such as the way of production and the specification of extracts.

A consistent quality of herbal drugs may need more detailed information on aspects of agricultural production. The selection of seeds, conditions of cultivation and harvesting represent an important aspect in producing a reproducible quality of herbal drugs. Ongoing discussions on Good Agricultural Practices (GAP) for medicinal plants should be monitored regularly [5].

Significances of medicinal plants to human being

- Many of the modern medicines are produced indirectly from medicinal plants for example Aspirin
- Plants are directly used as medicines by a majority of cultures around the world. For example, Chinese medicine and Indian medicine
- Many food crops have medicinal effects. For example, Garlic
- Medicinal plants are resources of new drugs. It is estimated that there are more than 250,000 flower plant species.
- Studying medicinal plant helps us to understand plant toxicity and protect human and animals from natural poisons.
- Cultivation and preservation of medicinal plants protect biological diversity. For example metabolic engineering of plants [6]

Inflammation

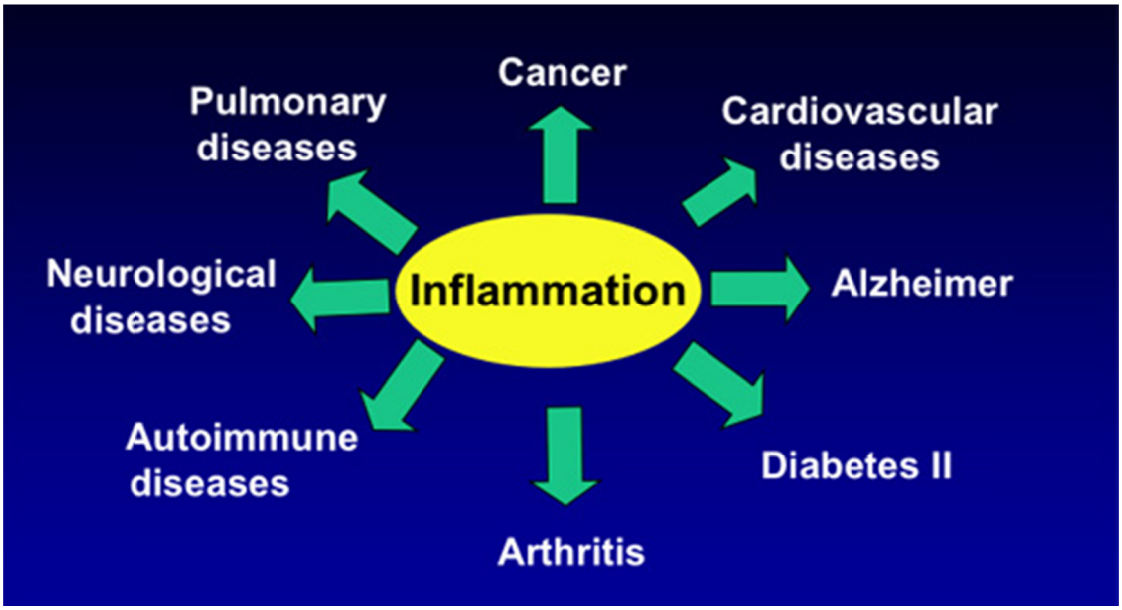
Inflammation is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens damaged cells, or irritants [7]. The classical signs of acute inflammation are pain, heat, redness, swelling and loss of function. Inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process.

Inflammation is not a synonym for infection, even in cases where inflammation is caused by infection. Although infection is caused by a microorganism,inflammation is one of

the responses of the organism to the pathogen. However inflammation is a stereotyped response, and therefore it is considered as a mechanism of innate immunity as compared to adaptive immunity, which is specific for each pathogen [8].

Progressive destruction of the tissue would compromise the survival of the organism. However chronic inflammation can also lead to a host of disease, such as hay fever, periodontitis , atherosclerosis, rheumatoid arthritis and even cancer (eg: gallbladder, carcinoma). It is for that reason that inflammation is normally closed regulated by the body.

Fig:1: INFLAMMATORY DISEASES



Classification

- Acute inflammation
- Chronic inflammation

Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system and various cells within the injured tissue.

Prolonged inflammation known as chronic inflammation leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process.

Table:1: Comparison between acute and chronic inflammation

CAUSES	ACUTE INFLAMMATION	CHRONIC INFLAMMATION
Causative agent	Bacterial pathogens, injured tissues	Persistent acute inflammation due to non- degradable pathogens, viral infection, persistent foreign bodies or acute immune reactions.
Major cells involved	Neutrophils(primary) basophils (in inflammatory response) and eosinophila (response to helminth worms and parasites) monocellular cells (monocytes, macrophages)	Monocellular cells(Monocytes, macrophages, lymphocytes, plasma cells) fibroblasts
Primary mediators	Vasoactive amines, eicosanoids	IFN –Y and other cytokines, growth factor, reactive oxygen species, hydrolytic enzymes
Onset	Immediate	Delayed
Duration	Few days	Upto many months or years
Outcomes	Resolution, abscess formation, chronic inflammation	Tissue destruction, fibrosis, necrosis

The five cardial signs of acute inflammation “PRISH”

PAIN – The inflamed area is likely to be painful, especially when touched. Chemicals that stimulate nerve endings are released, making the area much more sensitive.

REDNESS – This is because ofthe capillaries are filled up with more blood than usual.

IMMOBILITY – There may be some loss of function

SWELLING – Caused by an accumulation of fluid.

HEAT – As with the reason for the redness, more blood in the affected area makes it feel hot to the touch.

The five classical signs of inflammation

- Dolar – Latin term for pain
- Calor – Latin term for heat
- Rubor – Latin term means redness
- Tumor – Latin term for swelling
- Functiolaesa – which in Latin means injured function which can also mean loss of function [9].

Fig:2: INFLAMMATORY RESPONSES

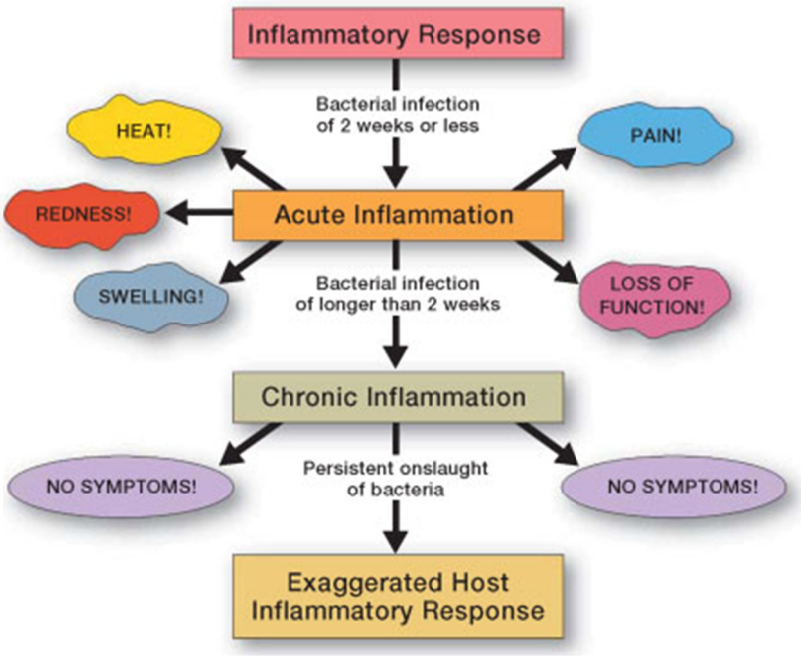


Table:2: Chemical mediators of the inflammatory response [10]

RESPONSE	MEDIATORS
1.Vasodilation	Prostoglandings (PG) PGI_2, PGE_1, PGE_2, PGD_2, Nitric oxide
2. Increased vascular permeability	Histamine C39, C5a (complement components) Bradykinin, Leukotrienes (LT), especially LTC_4,LTD_4, LTE_4, Platelet – activating factor, Substance – P, calcitonin gene – related peptide(CGRP)
3.Chemotaxis and leukocyte activation	C5a, LTB_4, lipoxins (LX), LXA_4, LXB_4, Bacterial products.
4.Tissue damage	Neutrophill and macrophages lysosomal products oxygen radicals
5.Fever	Interleukin – 1 (IL – 1), IL – 6, tumor necrosis fever (TNF), PGE_2, PGI_2, LTB_4, LXA_4, LXB_4
6.Pain	PGE_2, PGI_2, LTB_4, Bradykinin, CGRP

FIG:3: INFLAMMATION

Anti – inflammatory herbs and species

- Black pepper
- Basil
- Cardamom
- Chamomile
- Chives
- Cinnamon
- Cloves
- Garlic
- Ginger
- Parsley
- Nutmeg
- Rosemary
- Turmeric [11]

The extracts of Achillea millefolium, Artemisia vulgaris, Bauhinia tarapotensis, Curcuma longa, Forsythia suspense, Houttuynia cordata, Glycyrrhiza uralensis, Lonicera japonica and ValerianaWallichi have show anti- inflammatory activity [12].

Antibacterial activity

An antimicrobial is an agent that kills microorganisms or inhibits their growth. Antimicrobial medicines can be grouped according to the microorganisms they act primarily against. For example, antibacterial (commonly known as antibiotics) are used against bacteria and antifungal are used against fungi. Antimicrobials that kill microbes are called microbicidal. Those that merely inhibit their growth are called microbiostatic. Disinfectants such as bleach are non- selective antimicrobials [13].

An infection and immunity involve interaction between the animal body (host) and the infecting microorganism. Based on their relationships to their hosts, microorganism can be classified as saprophytes (from Greek sapros decayed; and phyton plant) and parasites.

Saprophytes are free - living microbes that subsist on dead or decaying organic matter. They are found in soil and water and play an important role in the degradation of organic materials in nature.

Parasites are microbes that can establish themselves and multiply in hosts. Parasitic microbes may be either pathogens (from Greek, pathos- suffering and gen- produce i.e disease- producing) or commensals (from Latin, com – with and mensa-living together).

Pathogens are microorganisms that are capable of producing disease in the host. Commensal microbes live in complete harmony with the host without causing any damage to it. The normal bacterial flora of the body consists largely of commensals. Many commensals behave as facultative pathogens in that they can produce disease when the host resistance is lowered.

Infections may be classified in various ways

- Initial infection with a parasite in a host is termed *primary infection*
- Subsequent infections by the same parasite in the host are termed *reinfections*.
- When a new parasite sets up an infection in a host whose resistance is lowered by a preexisting infectious disease, this is termed *secondary infection*
- *Focal infection* (more appropriately focal sepsis) indicates a condition where, due to infection or sepsis at localized sites such as the appendix or tonsils generalized effects are produced.

- When in a patient already suffering from a disease, a new infection is set up from another host or another external source it is termed *cross-infection*.
- *In-apparent infection* is one where the clinical effects are not apparent. The term *subclinical infection* is often used as a synonym.
- *Atypical infection* is one in which the typical or characteristic clinical manifestation of the particular infectious disease are not present [14].

Infections may be divided into following

- Bacterial infections
- Fungal infections
- Parasitic infections
- Protozoan infections
- Viral infections
- Worm infections

Bactrial infections

Pathogenic bacteria are bacteria which causes bacterial infections. Although the vast majority of bacteria are harmless or beneficial, quite a few bacteria are pathogenic. The highly pathogenic bacteria are classified in to two types “gram positive bacteria” and “gram negative bacteria”. The pathogenic bacteria contribute to globally important diseases such as pneumonia, which are caused by bacteria such as streptococcus and pseudomonas and food borne illness such as tetanus, typhoid fever, diphtheria, syphilis, and leprosy [15].

Gram positive bacteria	Gram negative bacteria
Staphylococcus aureus *Basillus subtilis* *Streptococcus viridians,* *S. pyogens*	*Esherichia coli* *Klebsiella* *Pseudomonas aeruginosa* *Salmonella typhi* *Proteus albus*

Major characteristics of microorganisms fall into the following categories

- **Morphological characteristics** cell shape, size and structure; cell arrangement; occurrence of special structures and developmental forms; staining reaction and motility and flagellar arrangement.
- **Chemical composition** - The various chemical constituents of the cells
- **Cultural characteristics** - Nutritional requirements and physical conditions required for growth and the manner in which growth occur.
- **Metabolic characteristics**-The way in which cells obtain and use their energy to carry out chemical reactions and regulate those reactions
- **Antigenic characteristics** - Special range chemical components (antigens) of the cell, distinctive for certain kinds of microorganisms.
- **Genetic characteristics** - characteristic of the hereditary material of the cell (DNA) and occurrence and function of other kinds of DNA that may be present, such as plasmids.
- **Pathogenecity**- The ability to cause disease in various plants or animals or even other microorganisms.

- **Ecological characteristics -** Habitat and the distribution of the organisms in the nature and the interactions between and among species in natural environments [16].

Mechanism of action of antimicrobial drugs

Antimicrobial agent may act by destroying the organism (bactericidal) or by inhibiting its growth (bacteriostatic). Bactericidal drugs in general are most effective against rapidly multiplying bacteria, often a bacteriostatic drug in higher concentration may act as bactericidal.

The selective toxic action on the infecting organism is the key to beneficial actions of antibiotics.These drugs can hit multiple targets in bacterial cell.

- The cell wall
- The cytoplasmic membrane
- The ribosomes
- The RNA molecules involved in transcription of genetic formation
- Enzymes required for DNA synthesis replication
- Metabolic pathways.

Antimicrobial agents

Antimicrobial drugs are effective in the treatment of infections because of their selective toxicity (i.e they have ability to kill or inhibit the growth of an invading microorganism without harming the cells of the host). In most instances, the selective toxicity is relative, rather than absolute, requiring that the concentration of the drug be carefully controlled to attack the microorganism while still being tolerated by the host. Selective antimicrobial therapy takes advantage of the biochemical differences that exist between microorganisms and human beings.

Agents used to treat bacterial infections

- Penicillin
- Cephalosporin
- Tetracyclines
- Aminoglycosides
- Macrolides
- Fluoroquinolones [17].

REVIEW OF LITERATURE

CHAPTER-II

LITERATURE REVIEW

The literature review of *Cordia obliqua willd*. and its related species reveals that the following works have been done on the plant. But the proper scientific studies have not been reported for the plant *Cordia obliqua*. As there is no pharmacognostic, phytochemical and pharmacological work of this traditionally much valued drug, the present work was taken up with a view to lay down the standards, which could be useful to detect the authenticity of this underutilized medicinally useful plant.

CORDIA OBLIQUA

VK. Agnihorti *et al*., (1987) have reported the chemical composition of the seeds of *Cordia obliqua* and isolated and characterized the presence of alpha- amyrin, betulin, octacosanol,lupeol- 3- rhamnoside, bêta – sitosterol, beta – sitosterolul- 3- glucoside, hentricontanol, hentricontane, taxifolin- 3, 5 –di rhamnoside and hesperetin- 7- rhamnose. The anti- inflammatory activity of the isolated compounds were determined.[18].

N.K. Udayaprakash., (2013) have studied the presence of phytochemicals in the aqueous extract of 100 different plants species including *Cordia* belonging to 44 families collected from Chennai, India.[19]

R.R.A. Abou- Shaaban *et al*., (2007) have reported the comparative pharmacological activity of cordia fruit mucilage at different stages of maturity, to determine the stage at which active substances were present in high propotions. Mucilage from both ripe and unripe *Cordia obliqua* decreased rabbit blood pressure and stimulates the respiratory rate.[20]

K. Thirupathi *et al*., (2008) have studied the pharmacological activities with extracts and purified compound indicates that the plants of cordia species possess analgesic, anti-inflammatory, anti microbial, anti viral, anti- fertility activities.[21]

Cordia malleodii

S.K. Sen *et al*., (2005) have reported that the leaves of *Cordia malleodii* plant, when crushed and applied on a fresh cut injury, bleeding stops forth with and the injury heals up quickly.[22]

Cordia sebestena

S. Jeera *et al*., (2011) have studied the phytochemical constituents and anti-bacterial activity of the flower extracts of *Cordia sebestena.*[23]

J. Dai *et al*., (2010) have studied the bio assay guided fractionation of an extract prepared from the fruits of *Cordia sebestena* led to the isolation of sebestenoids A-D (1-4).The fruits of *Cordia sebestena* yielded four phenyl propanoid esters, sebestenoids A-D (1-4). [24]

Cordia piauhensis

G.M.P. Santiago *et al*., (2005) have reported the larvicidal activity of the four monodesmoside saponins isolated from Penaclethra macroloba (Boraginaceae) and one bidesmoside saponin from *Cordia piauhensis* on 3rd instar larvae of Aedes aegypti.[25]

Cordia alliodora

A.K. Sinha *et al*., (2003) have synthesized methyl 2, 4, 5-trimethyl phenyl propionate by esterification of phenyl propionic acid with methanol, a metabolite of *Cordia alliodora* (94 percent yield within 3 minutes) by microwave assisted technique.[26]

Kloucek *et al*., (2007) have reported that the ethanol extract of stem bark of *Cordia alliodora* was effective against nine bacteria & one yeast using the broth micro dilution method. [27]

J.R. Loset *et al* ., (2000) have reported that the root bark of *Cordia alliodora* have anti fungal properties against the pathogenic mold elodesporium cucumerinum and a marked activity against larvae of the yellow fever transmitting mosquito Aedes aegypti.[28]

S. Kaur *et al*., (2010) have isolated 1,3-methoxy propanoyl-2, 4, 5-trimethoxy benzene from *Cordia alliodora*. Methoxy propanoyl trimethoxy benzene was reported to have antifungal and larvicidal activities against the phytopathogenic mould cladasporium cucumerinum and the larvae of yellow fever transmitting mosquito, Aedes aegypti respectively.[29].

Cordia verbenaceae

JS. Chaves *et al*., (2008) have reported a quantitative study to assess the plasma and tissue levels, tissue distribution and skin (ear) absorption of the sesquiterpene, alpha-humulene, the main active constituent isolated from the plant *Cordia verbenaceae* (Boraginaceae), after oral, intravenous and topical administration in mice. The alpha-humulene levels in tissues were quantified by GC-MS analysis. Its high amount was found in the liver followed by the kidneys, heart, lungs, spleen and brain, 0.5 hour after oral administration.[30]

F. RoldaoEde *et al*., (2008) have reported the antiulcer activity of *Cordia verbenaceae* extract which was evaluated using ethanol & piroxicam-induced gastric lesions method. Analgesic activity was evaluated by writhing, tail-flick & hot plate method. Antioxidant activity was determined by in vitro lipoper-oxidation assay.[31]

DM. De Oliveira *et al*., (2011) have reported that the *Cordia verbenaceae* inhibit the in-vitro secretion of histamine from mast cells of different animal species as well as the secretion of mast cells from animals treated with the extract.[32]

A.A.M. Oliveira *et al*., (1998) have reported the oral administration of the hydro alcoholic extracts of *Cordia verbenaceae* leaves in rats after 30 days caused no changes of serum parameters, cardiac function & toxic effect.[33]

Cordia dichotoma

D.N. Khairnar., (2006) have reported the medico-ethnological data regarding the specific parts of the different medicinal plants together with the ailments that can be treated and the specific uses of this plants in preventive, promotion and curative applications obtained from the tribal's well acquainted with medicinal plants *Cordia dichotoma* (Boraginaceae).[34]

Sumitra Singh *et al*., (2008) have reported the analgesic activity of different extracts of*Cordia dichotoma* (Boraginaceae) studied in Swiss albino mice.[35]

I.J. Kuppasta *et al*., (2003) have studied the in vitro anthelmintic activity of the butanol fractionated extract of the *Cordia dichotoma* against earth worms, tape worms and round worms.[36]

B.A.Jadeja *et al*.,(2008) have reported phonological observations of *Cordia dichotoma* (Ehretiaceae) on leaf- fall, new foliage, flowering and fruiting.[37]

Cordia myxa

Shraddhasuman *et al*., (2009) have reported *Cordia myxa* (Boraginaceae) bark mixed with water and filtered is given in chronic fever[38]

MG. Rajesh *et al*., (2000) have reported ten cirrhotic patients were given kamilari (one tab 850 mg, 3 times daily) for 4 months and biochemical parameters analysed in serum. Levels of serum enzymes, cholesterol and billirubin are decreased and that of proteins increased. It can be concluded that kamilari is a hepatoprotective drug composing of *Cordia myxa*. [(39)]

Cordia mutispicata

M. Das Gracas *et al*., (2010) have reported the essential oils obtained from five samples of *Cordia multispicata* collected in four municipalities of the state of Par (Brazil) by hydro distillation method and analysed by GC/FID and GC-MS. [(40)]

T.B. Correia Desilva *et al*., (2010) have reported the total phenolic content and antioxidant activity of extracts and four flavonoids isolated from the leaves of two boraginaceae species *Cordia multispicata* and Tournefortia bicolor which were evaluated using folin-ciocalteu reagent, DPPH free radical scavenging and inhibition of peroxidation of linoleic acid by FTC method.[(41)]

Cordia globosa

JP. David *et al* ., (2007) have reported that the *Cordia globosa* extract from the Brazilian northeastern semi-acid region , were evaluated through DPPH assays β-carotene bleaching & brine shrimp lethality tests.[(42)]

S. Jane Eire S *et al*., (2006) have reported the chemical composition of the essential oils obtained from the fresh leaves of *Cordia globosa* (Boraginaceae) at different ontogenetic stages and which were analysed by GC/ GCMS.[(43)]

Cordia latifolia

B.S. Siddiqui B.S *et al*., (2010) have isolated 3 new natural constituents from the fruits & leaves of *Cordia latifolia.*[44]

Cordia lucocephala

Jaecio Carlos Diniz *et al*., (2008) have isolated essential oil from the leaves of *Cordia lucocephala* by hydro distillation method which was subsequently analysed by GC/MS &GC-FID. [45]

Cordia linnaei

JR. Loset *et al*., (1998) have isolated 3 new meroterpenoid naphthoquinones, the known cordia quinine B & a new naphthoxirene from the roots of *Cordia linnaei*. The naphthoquinones showed activity against cladosporium cucumerinum, candida albicans & the larvae of the yellow fever transmitting mosquito Aedes aegypti.[46]

Cordia fragrantissima

K. Mori *et al*., (2008) have reported that the methanolic extract of the wood of *Cordia fragrantissima* exhibit significant activity against leishmania [47]

Cordia americana

GF. Mourasss-Costa GF *et al*., (2012) have reported that the extract of *Cordia americana* is most effective against herpes simplex virus type 1.[48]

Cordia curassavica

Tzasna Hernandez *et al*., (2007) have reported the anti microbial activity of *Cordia curassavica* against 13 bacteria & 5 fungal strains.[49]

Cordia spinenscens

Yasmina Auralim *et al*., (1997) have reported that the aqueous extract of *Cordia spinenscens* leaves contains magnesium lithospermato, calcium rosmarinate & magnesium rosmarinate and which act as potent inhibitory substances against HIV-1 reverse transcriptase.(50)

Cordia salicifolia

Matsunaga *et al*., (1997) have reported that the ethyl acetate extract of *Cordia salicifolia* showed potential negative inotropic effect on the isolated left atrium. (51)

Cordia trichotoma

S.A. Jane Eire *et al*., (2005) have isolated essential oils from the heart wood and sapwood of *Cordia trichotoma* (Boraginaceae) and whichwere analysed by GC/FID and GC/MS. (52)

Cordia gelletii

P.N. Okusa *et al*., (2007) have reported that the methanolic extract of root bark of *Cordia gelletii were* used for the treatment of various disorders, including malaria, diarrhoea, wounds and skin diseases and these activities may be due to antimicrobial and antioxidant properties.(53)

Cordia globifera

Dettrakul.S *et al*.,(2009) have isolated cordial globiferin from the root extracts of *Cordia globifera*. Antimalarial, antifungal and cytotoxic activities were also evaluated. (54)

AIM AND SCOPE

CHAPTER - III

AIM AND SCOPE OF THE STUDY

Plants have been used for healing purpose and form the origin of much of the modern medicine. Many medicinally important drugs originate from plant sources. The production of drugs from plants continues and many pharmaceutical companies engaged in large- scale pharmacological screening of herbs.

Cordia obliqua willd. is an important medicinal plant, belonging to the family Boraginaceae. It is a small deciduous tree, having ovate leaves and white flowers [(55)].

The ethnomedical information states that almost all parts of this tree are employed for a number of disorders.

The leaves are useful in ulcers and in headache. The juice of the bark along with coconut oil is given in gripes. The barks and also the unripe fruit are used as a mild tonic. Fruits are used as anthelmintics, astringent, demulcent, diuretic, expectorant in bronchial affections, irritation of urinary passages. The kernals are a good remedy in ringworm. The santals use the powder of the bark as an external application in prurigo. The Javanese use the bark in fever [(56)].

The Phytochemical studies of *Cordia obliqua* willd. have revealed the presence of steroids, alkaloids, triterpenes, phenolic compounds, flavonoids and reducing sugars [(57)].

Based on the above information and studies available, the present research work has been framed to carry out the following studies on the leaves of *Cordia obliqua* wild.

1. Pharmacognostical studies

Morphological evaluation

Microscopical analysis

Physio- chemical parameters

Leaf constants

2. Phytochemical studies

PreliminaryPhytochemical screening

Fluorescence analysis of powder

Quantitative estimation of phytoconstituents

Chromatography (TLC, HPTLC) method

3. Pharmacological studies

Anti- oxidant activity by invitro methods such as

•DPPH assay

•Ferric reducing antioxidant power assay

•Phosphomolybdenum method

•Nitric oxide scavenging activity

•Reducing power assay

Anti- inflammatory activity by invivo method – Carrageenan induced paw oedema method.

Anti- bacterial activity using organisms such as staphylococcus aureus, streptococcus alpus, pseudomonas, E.coli.

PHARMACOGNOSTICAL STUDIES

Fig .4: HERBARIUM of *Cordia obliqua willd*

CORDIA OBLIQUA, *WILLD.*

CHAPTER – IV

PHARMACOGNOSTIC STUDIES

SECTION – A

GENERAL DESCRIPTION OF THE PLANT

Cordia obliqua willd.is a medium sized deciduous tree with moderately broad branches.

Botanical source :*Cordia obliqua*

Family : Boraginaceae

Synonyms : Cordia wallichii G. Don.,

: Cordia myxa Linn.,

: Cordia latifolia Roxb.

Common name : Clammy cherry, Mookuchalipazham

Vernacular names

English - Large sebesten

Hindi - Baralessura

Sanskrit - Bhukarvudara

Kanada - Chellamara, nakkera

Malayalam - Pasakamaram,

Telugu - Nakkera

Tamil - Naruvili

Taxonomic classification [58]

Kingdom - Plantae

Subkingdom -Tracheobionta

Super division - Spermatophyta

Division - Magnoliophyta

Sub class - Asteridae

Order - Lamiales

Family - Boraginaceae

Genus - *Cordia*

Species - *obliqua*

Geographical Distribution (Habitat) (Fig. 5)

Widely spread over the whole of the warmer parts of India and Ceylon (often cultivated). Malacca-Indo-china, Hainan, Formosa, Java, Phillipines, New Guinea, Australia [58]. Common throughout India, particularly in the western region [59].

Description

Tree

Cordia obliqua Willd. is a medium sized tree,10.5m high, found scattered throughout the mid- Himalayas up to elevations of 1,470 meters.

There are two forms of cordia obliqua willd., occurring in Himachal Pradesh. The major difference between these two is the size of the fruits, which is small in one case and large in the other. The present observations were recorded only on the small- fruited type which is common.

Leaves (Fig.6)

Leaves are alternate, ovate, 10.1cm long, 5.7cm broad, entire to slightly dentate with pinnately- reticulate venation.Young leaves are tomentosefrom beneath and the matured leaves are glabrous, but more or less rough when full grown. Sometimes, variable in shape from elliptic lanceolate to broad ovate, often with a rounded or cordate base, basal nerves 3,rarely5,blade 3-6, petioles 2.5-5cm long.

Flowers (Fig.8)

Flowers are small and the average diameter of a fully open flower, 6mm, very short stalked, bisexual, white in colour. Inflorescence,terminal or an axillary cyme almost resembling a biparous cyme. Flowers per cluster,14. Calyx is cup shaped and gamosepalous, 4mm, green. Corolla, creamish white, polypetalous with 4 petals , 6mm. stamens 2in number, epipetalous with a very small filament. Gynoecium has a globose ovary and bifurcated.

Flowers from March- April and fruits from May-June.

Fruits (Fig.7)

Fruit is a drupe, 1.75cm in diameter, light yellow to slightly greenish in colour, with a light-red tinge at the time of full maturity; epicarp, thick; mesocarp, mucilaginous; endocarp, hard and stony.

Bark

Bark is dark greyish brown in colour, surface is rough with transverse and longitudinal cracks and fissures,inner surface deep greyish; fractured surface horny; taste and odour indistinct.

Parts used

Fruits, leaves and bark are used medicinally.

Collection of plant

The plant of Cordia obliqua willd. was collected from the Madurai medical college campus, Madurai and it was authenticated by Taxonomist. The leaves were washed thoroughly and dried in shade (Fig.4)

Fig. 5: HABIT AND HABITAT OF THE PLANT

Fig:6: LEAVES OF *Cordia obliqua willd*

Fig:7.1: Dorsal view

Fig:7.2: Ventral view

Fig .8: FRUITS OF *Cordia obliqua willd*

Fig.9: FLOWER OF *Cordia oblique willd*

SECTION – B

MICROSCOPICAL EVALUATION OF THE LEAVES [60-71]

The leaves of the plant were subjected to microscopical evaluation. The samples of leaves were cut and removed from the plant and fixed in FAA (formalin, 5ml; acetic acid, 5ml; ethyl alcohol, 90ml). After 24h of fixing, the specimens were dehydrated with graded series of t-butyl alcohol . Infiltration of the specimens was carried by gradual addition of paraffin wax (M.Pt-58-60^{o}C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

Sectioning

The paraffin embedded specimens were sectioned with the help of rotary microtome. The thickness of the sections was 10-12µm. De-waxing of the sections was carried out by customary procedure. The sections were stained with toluidine blue since it is a polychromatic stain. The staining results were remarkably good and some cytochemical reactions were also obtained. The dye rendered pink color to the cellulose walls, blue to the lignified cells, violet to the mucilage and blue to the protein bodies and also stained with safranin.

Leaf clearing

Two methods were used for studying the stomatal morphology, venation pattern and trichome distribution. Paraffin embedded leaf was used for para-dermal sections. From these sections, the epidermal layers as well as vein islets were studied. Another method employed was clearing leaf fragments by immersing the material in alcohol (to remove chlorophyll) followed by treating with 5% sodium hydroxide. The material was rendered transparent due to loss of cell contents. Epidermal peeling by partial maceration employing Jeffrey's

maceration was also done. Glycerin mounted temporary preparations were made for cleared materials.

For the study of elements of xylem, small fragments of leaves were macerated with Jeffery's maceration fluid.

Powdered materials of different parts were cleared with sodium hydroxide and mounted in glycerin medium after staining. Different cell components were studied and measured.

Photomicrographs

The photographs of different magnifications were taken with Nikon lab photo 2 microscopic units. For normal observations bright field was used and for the study of starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized they appear bright against dark background. The magnifications of the anatomical features are indicated by the scale-bars in the photographs.

The microscopic features of the leaf of the tree were presented in **Fig.9 to14**

MICROSCOPICAL STUDY OF THE LEAVES

Anatomy of the leaf

The transverse section of the leaf shows the following anatomical features

Leaf (Fig.9: 1,2)

The leaf is dorsiventral, xeromorphic and hypostomatic. The midrib is thick and wide and plano-convex in sectional view; the adaxial side is flat and the abaxial part is semicircular **(Fig.9.1)**. It is 900µm thick and 850µm wide. The epidermal layer is thin and the cells are spindle shaped, thick walled and darkly stained. The ground tissue around the vascular strand is parenchymatous; the cells are circular, thick walled and compact.

The structure of the vascular system is complex. It consists of abaxial wide, shallow bowl shaped vascular strand and two smaller adaxial vascular strands **(Fig.9.2)**. The abaxial vascular strand consists of several solitary, diffusely distributed xylem elements and thick walled lignified fibres. Along the lower part of the xylem strand, occur several discrete phloem masses. **(Fig.9.2)** The adaxial vascular strands are collateral having a few rows of xylem elements and phloem on the centre part of the xylem. The adaxial and abaxial vascular strands are surrounded by a thick sclerenchyma shealth.

Lamina (Fig.10.1)

The lamina exhibits dorsiventral symmetry. The adaxial epidermis is fairly thick walled; the cells cylindrical and have prominent cuticle. The abaxial epidermis has smaller, thin walled cells. The epidermis is stomatiferous **(Fig.10.1)**.

The mesophyll tissue is differentiated into adaxial band of palisade cells and abaxial zone of spongy parenchyma cells. The palisade cells are thin, elongated compact and

Fig.9.1 : T.S of leaf through Midrib

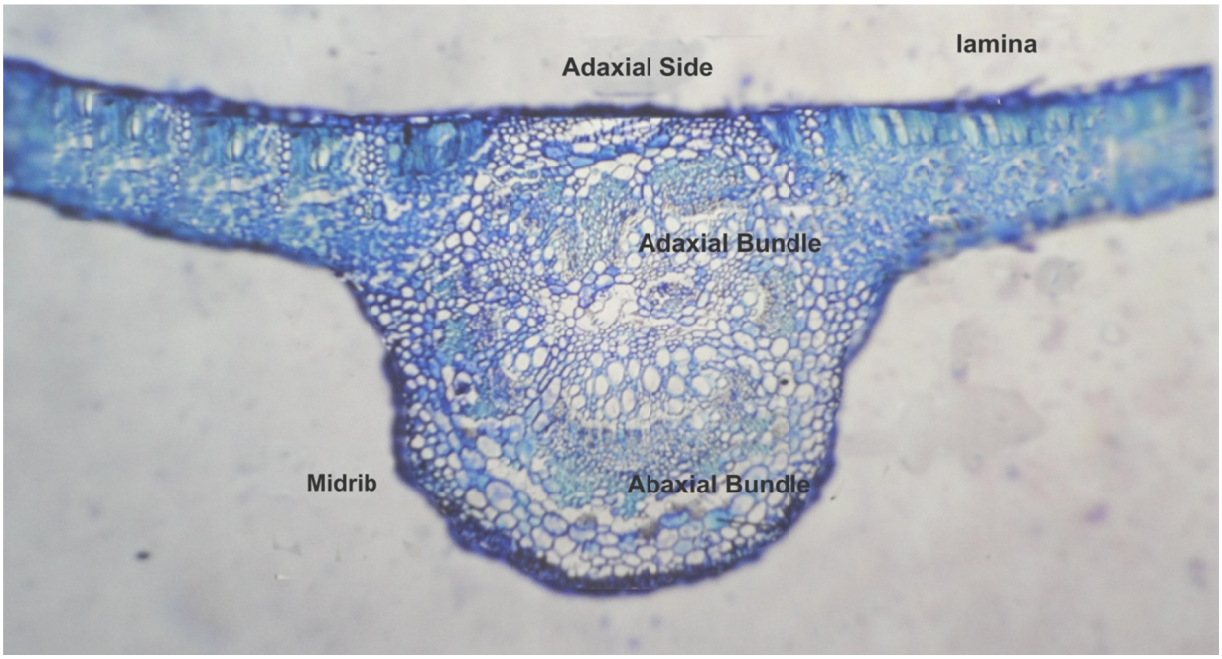


Fig.9.2: T.S of Midrib enlarged

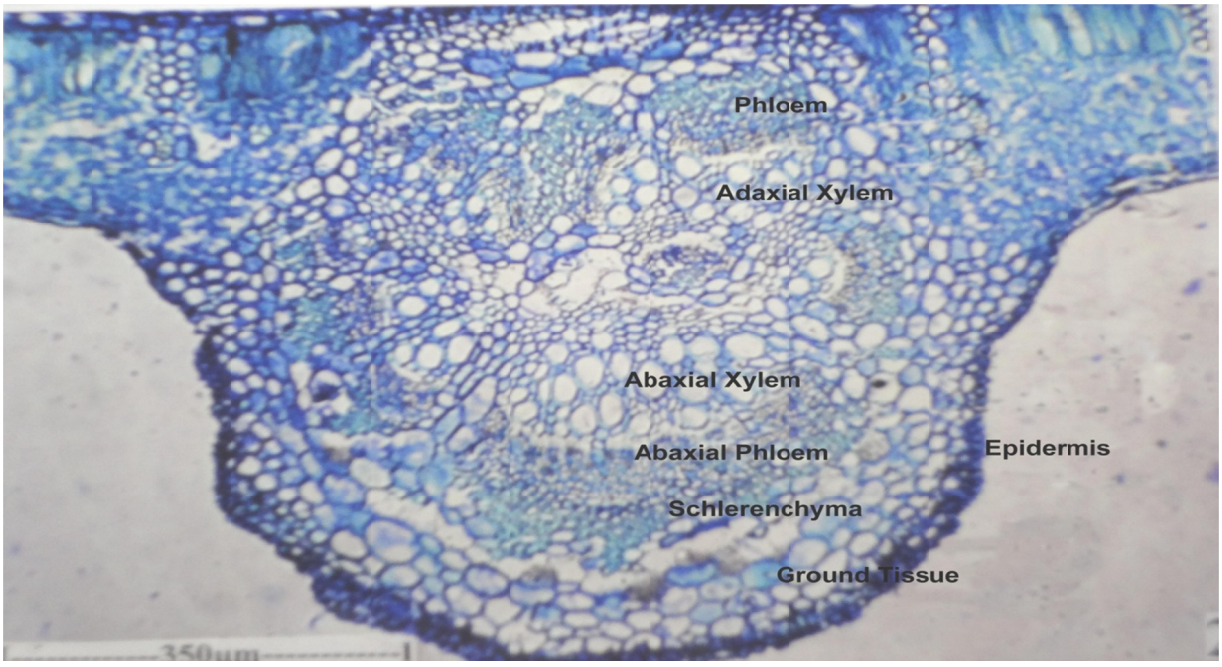


Fig.10.1 : T.S of Lamina

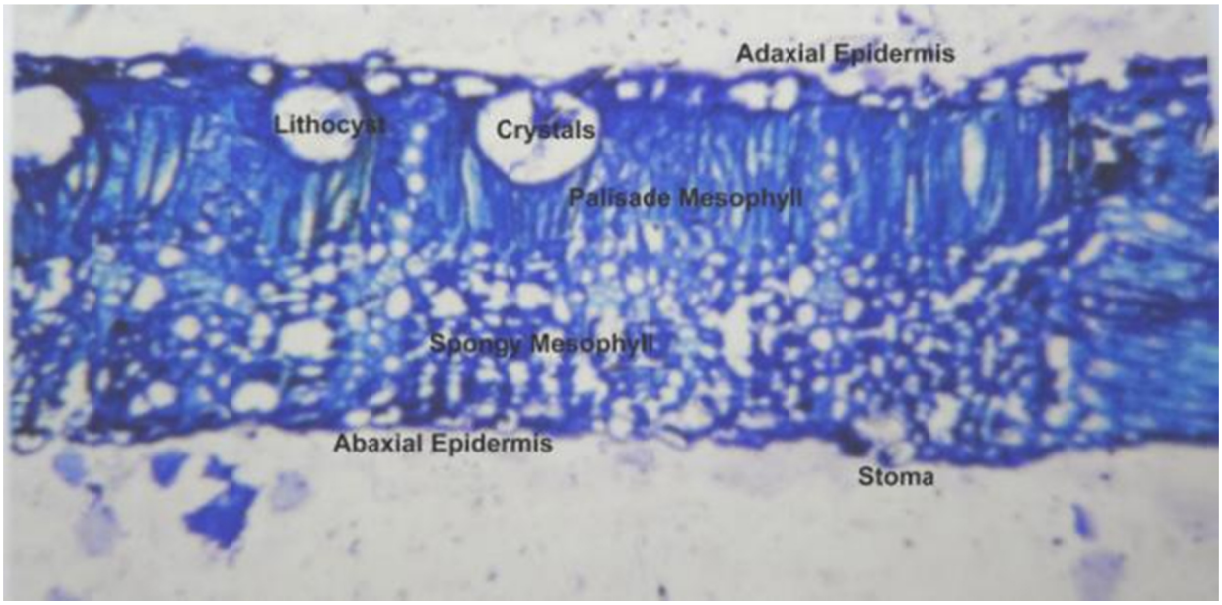


Fig.10.2 : Crystal distribution in the Midrib

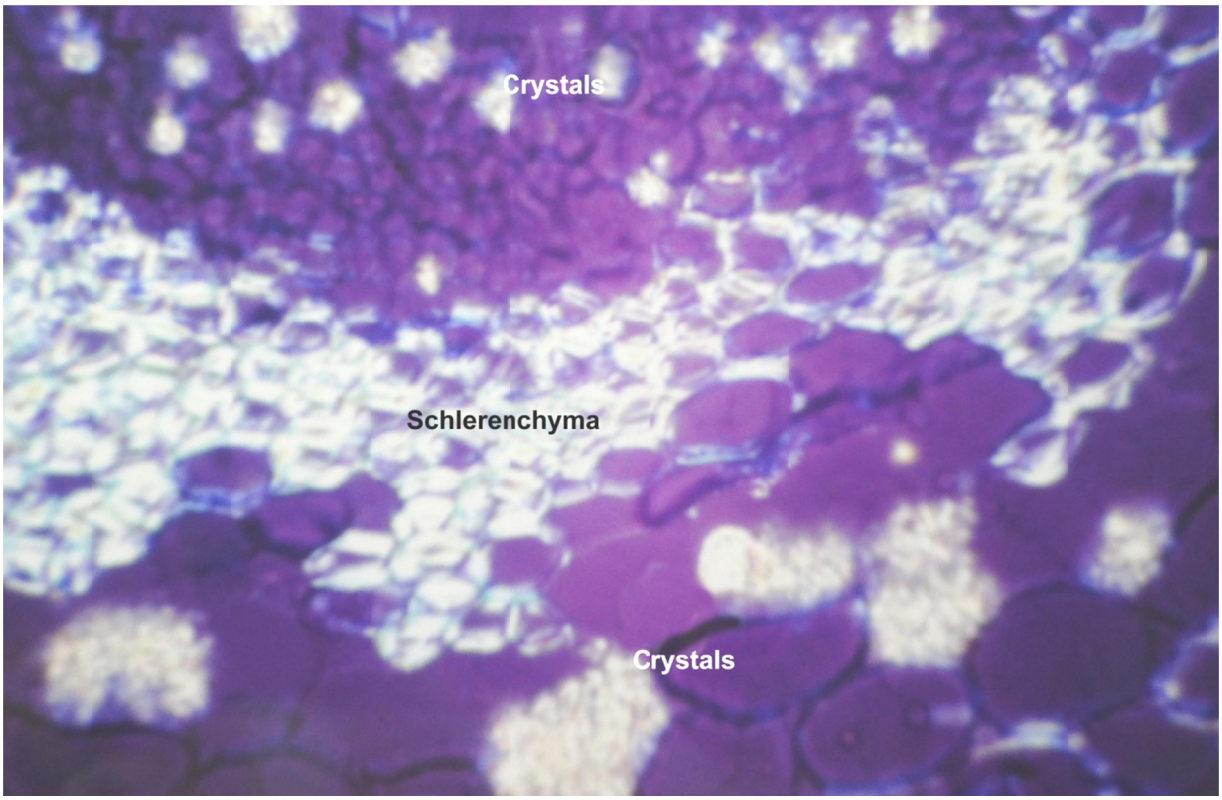


Fig. 11.1 : Venation Pattern

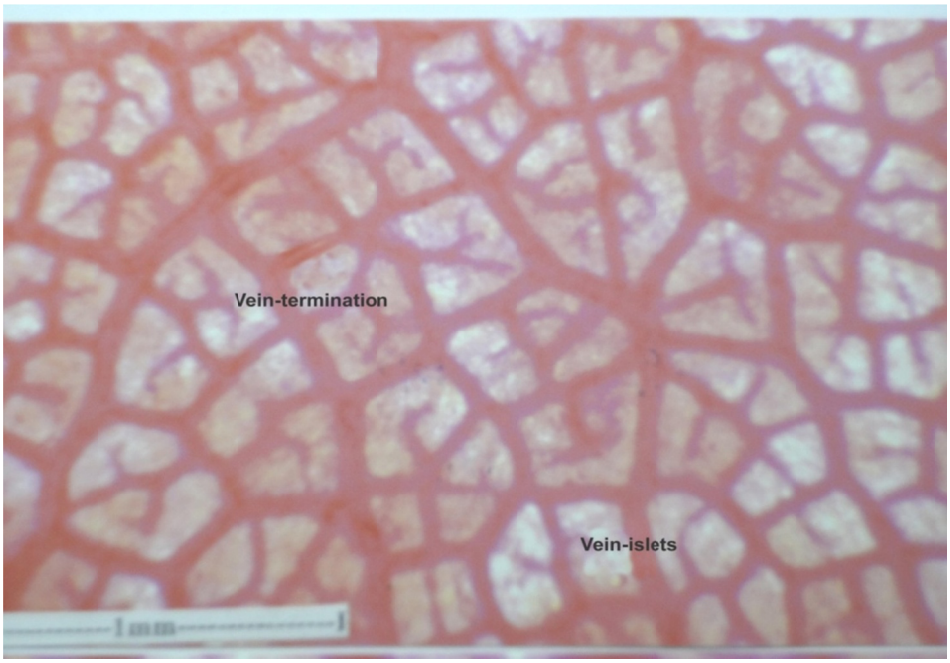
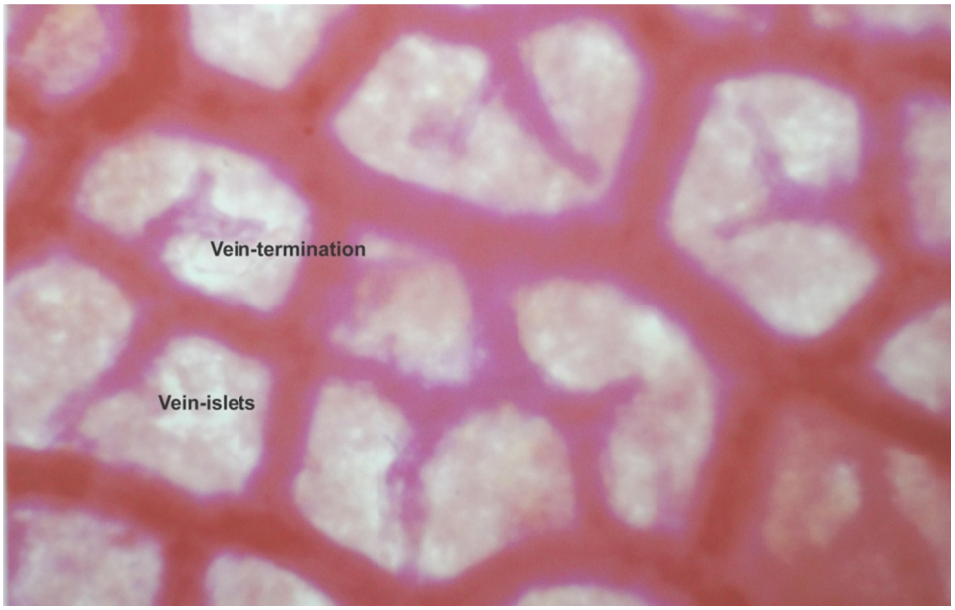


Fig.11.2. : Veins enlarged



chlorophyllous. The spongy parenchyma cells are spherical and lobed forming wide air-spaces **(Fig.10.1)**. The lamina is 200µm thick. The palisade cells are 80µm in height.

Crystals (Fig.10.2)

Two types of crystals are seen in the leaf.

1. Calcium carbonate crystals are located in specially modified epidermal cells of the leaf **(Fig. 10.1)**. The epidermal cells are lightly dilated into circular (spherical) cells called lithocysts; with in the lithocysts occur cystolith with a short stalk attached on the epidermal cell wall.
2. Calcium oxalate crystals are large granular bodies located in the ground parenchyma of the midrib and mesophyll cells **(Fig.10.2)**.

Venation pattern (Fig. 11. 1 & 2)

The veins and veinlets are thick and straight. The vein- islets are narrow, squarish or polyhedral in outline. The vein boundary is thick. The vein -termination are short and thick. They are simple, forked once or twice **(Fig.11.2)**.

Epidermal trichomes (Fig 12.1)

Unique types of epidermal trichomes are frequently seen on the abaxial epidermis. The trichome is nonglandular type. It consists of bulbous basal part measuring 80µm thick. The terminal part is sharp, pointed and thin. The trichome is 140µm long. It arises from epidermal pit. The surface of the trichome has echinate out growths.

Fig.12.1 : Bulbous epidermal trichome on the leaf

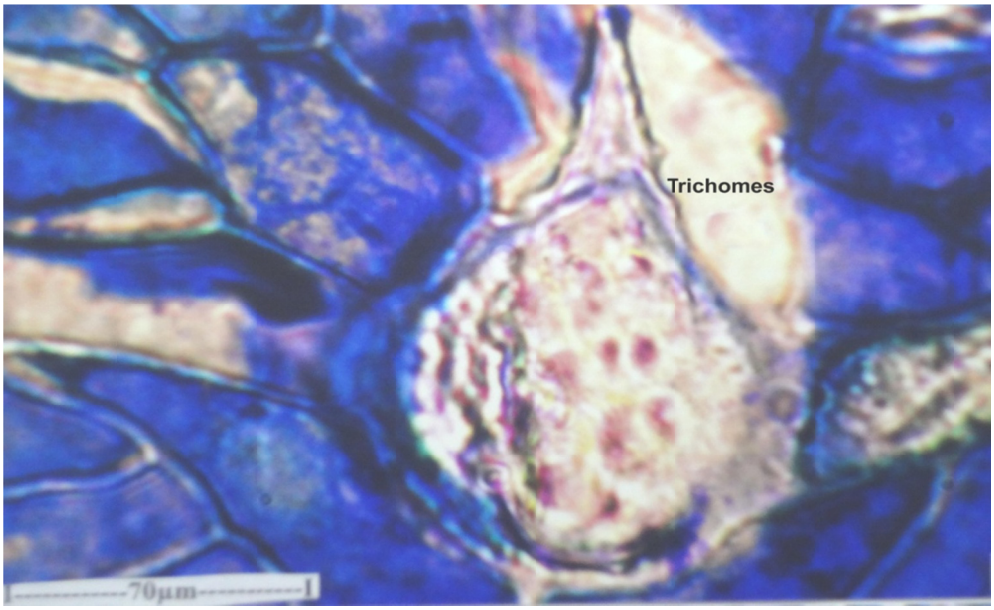


Fig.12.2. : Abaxial epidermal layer showing stomatal type

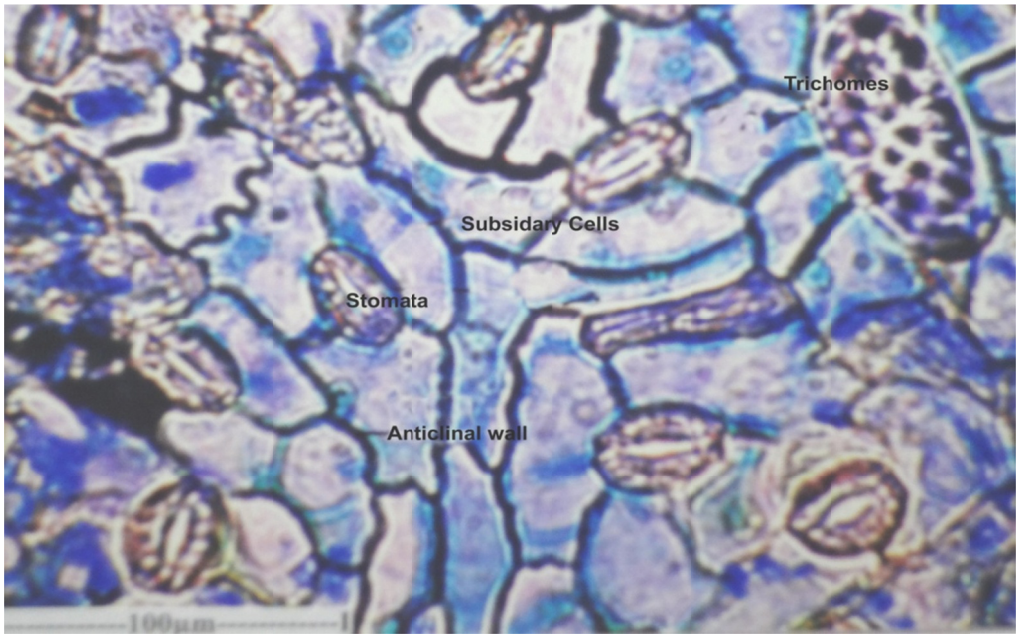


Fig.13.1 : Stomata in surface of the abaxial epidermis

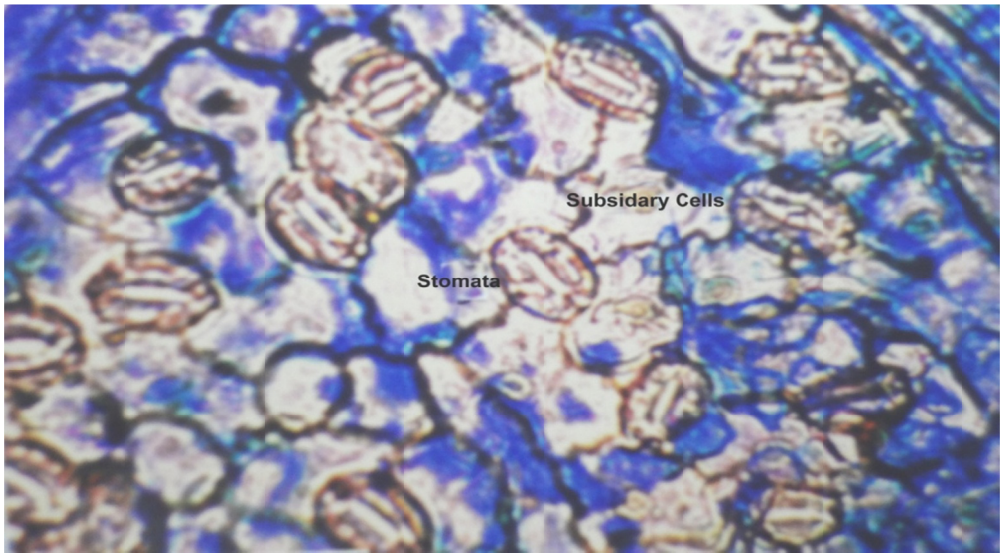


Fig.13.2 : Stomata enlarged

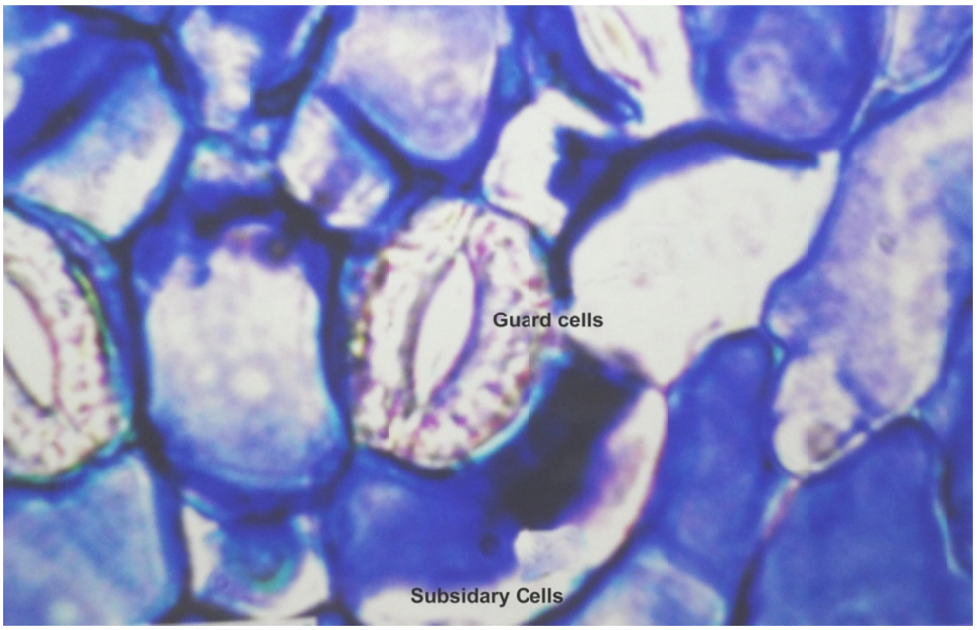


Fig.14.1 : Adaxial epidermis cells in surface view

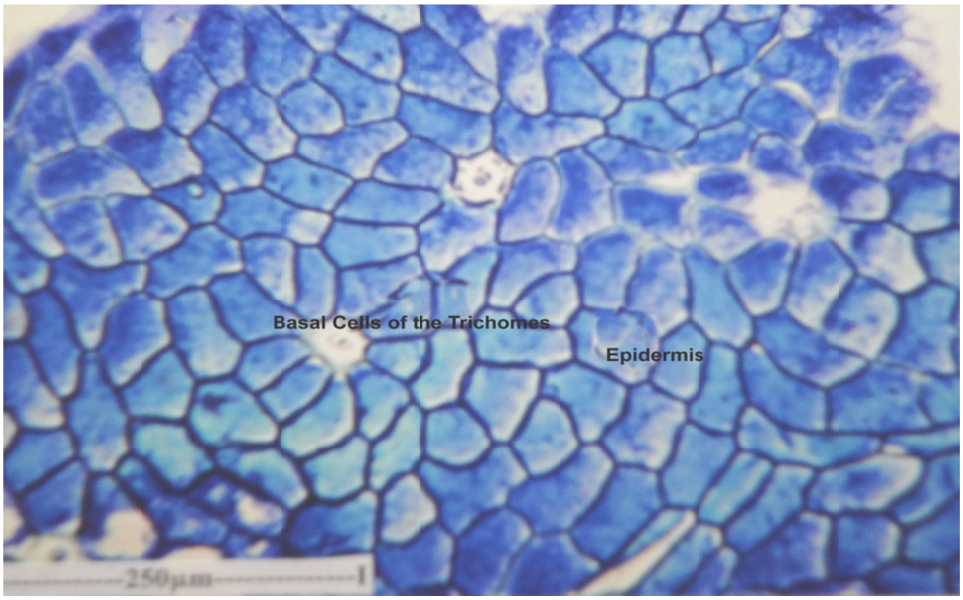
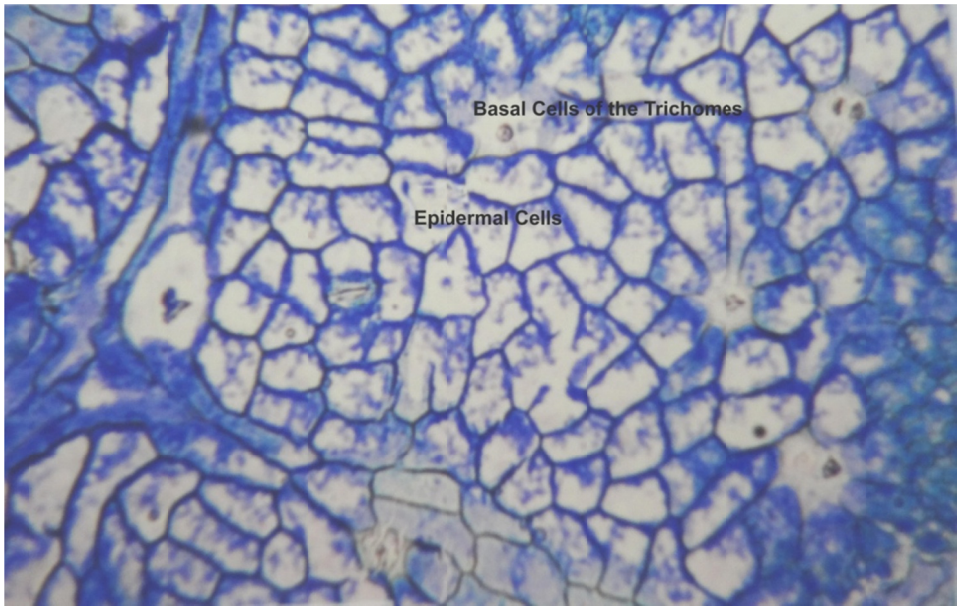


Fig.14.2: Adaxial epidermis cells in surface view



Stomata

Stomata occur on the abaxial epidermis only. The stomata are actinocytic, having three or four radiating subsidiary cells **(Fig.13.2 & 14.1,2)**. The epidermal cells are thick walled; the anticlinal walls slightly wavy. The stomata are 20 × 30μm in sizes.

The adaxial epidermis is apostomatic. The cells are polygonal with straight anticlinal walls **(Fig. 14.1 & 2)**. There are circular, shallow cavities on the epidermis from which the epidermal trichomes arises **(Fig 14.1)**

SECTION -C

QUANTITATIVE ANALYTICAL MICROSCOPY [72]

Quantitative analytical microscopy is useful for measuring the cell contents of the crude drugs, which help in their identification, characterization, and standardization. A clear idea about the identity and characteristic features of the drug can be obtained after several numbers of determinations; the characteristics number obtained was noted and compared with a standard value to find out whether it is within the range.

Determination of stomatal number and stomatal index

Stomatal number: The average number of stomata/sq.mm area of each surface of a leaf epidermisis termed as stomatal number [(73)].

Stomatal index: The stomatal index is the percentage which the number of stomata formed to the total number of epidermal cells, each stoma being counted as one cell.

To study the stomatal morphology (type of stomata), stomatal number and stomatal index of leaf, the leaf was subjected to epidermal peeling.

Procedure [(73)]

The leaf was cleared by boiling with chloral hydrate solution or alternatively with chlorinated soda. The upper and lower epidermis was peeled out separately by means of forceps. The cleared leaf was placed on a slide and mounted in glycerin. A camera lucida and drawing board was placed and a stage micrometer was inserted for making the drawing scale. A square of 1mm was drawn by means of stage micrometer. The slide with cleared leaf (epidermis) was placed on the stage of the microscope and examined under 45X objective and 10X eye piece. The epidermal cell and stomata was traced. The numbers of stomata present in the area of 1sq. mm. including the cell if at least half of its area lies within the

square was counted. The result for each field was calculated and the average number of stomata per sq. mm was determined and their values were tabulated in **Table3**.

For stomatal index, the glycerin mounted leaf peeling as mentioned above was made and circle (O) like mark for each stomata and a cross (X) like mark for each epidermal cells was marked on the chart paper. The stomatal index was calculated by using the formula,

Stomatal index = S/(E + S) x 100, where S was the number of stomata in 1sq mm area of leaf and E was the number of epidermal cells (including stomata) in the same area of leaf. The values were tabulated in **Table 4.**

Table:3: Stomatal number of the leaves of *Cordia obliqua willd.*

OBSERVATION NUMBER	LOWER EPIDEMIS
1	**57**
2	**68**
3	**62**
4	**64**
5	**79**
6	**53**
7	**59**
8	**63**
9	**65**
10	**55**
MINIMIUM	**53**
AVERAGE	**62.5**
MAXIMUM	**79**

Table:4: Stomatal index of the leaf of *Cordia obliqua* willd.

OBSERVATION NUMBER	LOWER EPIDERMIS
1	54.63
2	47.59
3	56.87
4	46.85
5	45.73
6	52.94
7	46.73
8	50.48
9	49.52
10	58.94
MINIMUM	45.73
AVERAGE	48.41
MAXIMUM	58.94

Determination of Vein Islets and Vein Terminations

The term vein islet is used to denote the minute area of photosynthetic tissue encircled by the ultimate divisions of conducting strands. The number of vein islet/sq.mm of leaf fragments is known as **vein islet number**. An ultimate free end or termination of a veinlet is termed as veinlet termination. The number of vein terminals present in one sq.mm area of leaf fragment is termed as **veinlet termination number** [(75)].

Procedure [74]

The fragment of leaf lamina with an area of not less than 1sq.mm excluding the midrib and the margin of the leaf was taken. The fragments of leaf lamina were cleared by heating in a test tube containing chloral hydrate solution on a boiling water bath until clear. The cleared fragments were stained with saffranin solution and a temporary mount was prepared with glycerol solution. The stage micrometer placed on the microscopic stage, examined under 10X objective and 6X eye piece and an area of 1 sq mm square was drawn. The cleared leaf piece was placed on the microscope stage, the vein islets and vein terminals included in the square was drawn. The number of vein islets and terminals within the square were counted. The results obtained for the number of vein islets and terminals in1sq mm were tabulated in **Table 5**.

Table:5: **Vein islet and vein termination number of *Cordia obliqua willd*.**

OBSERVATION NUMBER	VEIN ISLET NUMBER	VEIN TERMINATION
1	23	27
2	25	33
3	18	25
4	13	29
5	17	31
6	11	19
7	9	39
8	16	37
9	10	28
10	27	21
RANGE		
MINIMUM	9	19
AVERAGE	16.9	28.9
MAXIMUM	27	39

SECTION – D

PHYSICAL PARAMETERS

Determination of Ash values [75,76]

Ash Content

The residue remaining after incineration of the drug is the ash content of crude drug, which simply represents inorganic salts naturally occurring in the drug or adhering to it or deliberately added to it as a form of adulteration. The total ash, acid insoluble ash and water soluble ash are generally determined.

Procedure

Determination of Total Ash

An accurately weighed 2g of air dried powdered drug was taken in a tarred silica crucible and incinerated at a temperature not exceeding 450^{o}C, upto 6hrs until free from carbon then allowed to cool and weighed. The percentage of ash was calculated with reference to the air dried drug.

Determination of Acid Insoluble Ash

The total ash obtained from the previous procedure was mixed with 25ml of 2M hydrochloric acid and boiled for 5min in a water bath, and then the insoluble matter was collected in an ashless filter paper (Whatmann) and washed with hot water, dried and ignited for 15min at a temperature not exceeding 450^{o}C, cooled in a dessicator and weighed. The percentage of acid insoluble ash was calculated with reference to the air dried drug.

Determination of Water Soluble Ash

The total ash obtained from the previous procedure was mixed with 25ml of water and boiled for 5min in a water bath, and then the insoluble matter was collected in an ashless filter paper and washed with hot water, dried and ignited for 15min at a temperature not exceeding 450^{o}C, cooled in a dessicator and weighed. The insoluble matter was subtracted from the weight of the total ash; the difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried drug.

Determination of sulphated ash [77]

An accurately weighed 2g of air dried powdered drug was taken in a tarred silica crucible which was previously ignited and cooled before weighing at a temperature not exceeding 450°C. The residue was moistened with 1ml of concentrated sulphuric acid, ignited at 800 ± 25°C until all black particles have disappeared. It was then cooled; again sulphuric acid was added and ignited. It was cooled and the percentage of sulphated ash was calculated with reference to air dried drug.

The values in respect of the total ash values, acid insoluble ash and water soluble ash and sulphated ash value were tabulated in **Table 6 .**

Table:6: Ash values of the leaves of *Cordia obliqua willd.*

OBSERVATION NUMBER	TOTAL ASH%	ACID INSOLUBLE ASH%	WATER SOLUBLE ASH%	SULPHATED ASH%
1	10.5	0.84		0.89
2	12	0.90		0.85
3	11.5	0.90		0.90
4	9.5		0.94	
5	11.5		0.94	
6	8.86		0.90	
MINIMUM	8.86	0.84	0.90	0.85
AVERAGE	10.64	0.88	0.92	0.88
MAXIMUM	12	0.90	0.94	0.90

Loss on drying [75, 76]

The method described by Wallis was followed for the determination of loss on drying.

One gram of the powdered leaf was accurately weighed in a tarred dish and dried in an oven at 105ºc for one hour. It was cooled in a dessicator and again weighed. The loss on drying was calculated with reference to the amount of dried powder taken and presented in **Table. 7**

Table:7: Percentage of loss on drying

Observation number	Loss on drying (%) w/w
1	9.35
2	9.32
3	10.09
4	9.82
5	9.43
6	10.10
Minimum	9.32
Average	11.23
Maximum	10.10

Determination of Extractive Values

The extractive values are the important factor to determine the amount of active principle or phytocostituents present in the plant materials, when extracted with suitable solvents. The extraction of crude plant materials with various solvents gives a solution containing different phytoconstituents. Composition of the phytoconstituents in a particular solvent depends upon the nature of drugs and solvents used. This is an important tool for the analysis of crude plant materials for its identity, purity and quality.

Procedure

Determination of ethanol soluble extractive

An accurately weighed 5g of the air dried coarsely powdered drug was macerated with 100ml of ethanol in a closed flask for 24h, shaking frequently during the first 6hours and allowed to stand for 18hours. Thereafter filtered rapidly, taking precautions against loss of ethanol. Then evaporate 25ml of the filtrate to dryness in a tarred china dish at 105^{o}C and

weighed. The percentage of ethanol soluble extractive was calculated with reference to the air dried drug.

Determination of ethyl acetate soluble extractive:

The procedure adopted under ethanol soluble extractive was followed using ethyl acetate as a solvent.

Determination of chloroform soluble extractive:

The procedure adopted under ethanol soluble extractive was followed using chloroform as a solvent.

Determination of petroleum ether soluble extractive:

The procedure adopted under ethanol soluble extractive was followed using petroleum ether as a solvent.

Determination of acetone soluble extractive:

The procedure adopted under acetone soluble extractive was followed using acetone as a solvent

Determination of water soluble extractive:

An accurately weighed 5g of the air dried coarsely powdered drug was macerated with 100ml of chloroform water in a closed flask for 24hours, shaking frequently during the first 6hours and allowed to stand for 18hours. Thereafter filtered rapidly, taking precautions against loss of chloroform water. Then evaporate 25ml of the filtrate to dryness in a tarred china dish at105^{o}C and weighed. The percentage of water soluble extractive was calculated with reference to the air dried drug.

The extractive values obtained for different solvents were presented in **table 8.**

Table:8: Extractive values of *Cordia obliqua willd.*

S.No	Solvents Used (Increasing order of polarity)	Extractive Value (% w/w)
1	Petroleum ether	16.79
2	Ethyl acetate	1.19
3	Chloroform	2.95
4	Ethanol	2.67
5	Acetone	1.59
6	Water	18.22

Determination of Foaming Index [75]

Some plant materials when shaken with water cause persistent foam which may be attributed to the presence of saponins in that material. The foaming ability of an aqueous solution of plant materials and their extracts is measured in terms of foaming index.

Procedure

An accurate quantity of about 1g of the coarse plant material was weighed and transferred into an Erlenmeyer flask containing 100ml of boiling water. The flask was boiled at moderate heat for 30min. The solution was cooled and filtered into a 100ml volumetric flask and sufficient distilled water was added to make up the volume. The solution was poured into ten stoppered test tubes in successive portions of 1ml, 2ml, etc. upto 10ml and the volume of the liquid in each tube was adjusted with water upto 10ml. The tubes were then stoppered and shaken in a length wise motion for 15sec (two shakes/sec) and allowed to stand for 15min. The height of foam was measured. If the height of the foam in every tube was less than 1cm, the foaming index was less than 100. If the height of the foam, 1cm was measured

in any test tube, the volume of the plant material decoction in this tube (A) was used to determine the index. If the height of the foam was more than 1cm in every tube, the foaming index was over 1000. In this case, the determination was repeated using a new series dilution of the decoction in order to obtain a result. The foaming index was calculated by using the following formula 1000/A where A was the volume in ml of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm was observed. The results obtained was presented in **table 9**.

Determination of Swelling Index [76]

Swelling index is the volume in ml taken up by the swelling of plant material under specified conditions. The medicinal plant materials like gums, mucilage, and pectin have swelling property.

Method

An accurately weighed 1g of the powdered drug material was taken in the 25ml glass stoppered measuring cylinder. 25ml of water was added and shaken the mixture thoroughly every 10min for 1h. Then, allowed to stand for 3hrs at room temperature. The volume in ml occupied by the plant material was measured, including sticky mucilage. The results obtained were presented in **table 9.**

Table: 9: Foaming and swelling index of *Cordia obliqua willd.*

Content	
Foaming index	**< 100**
Swelling index	**3.66±0.81**

SECTION – E

POWDER MICROSCOPY

i). Fluorescence analysis

The fluorescent analysis of the plant extracts of *Cordia obliqua* was carried out by using the method of Chase and Pratt (1949) [78]. The observations were tabulated in **Table10**

Table:10: Fluorescence analysis of extracts of *Cordia obliqua willd.*

Extracts	Colour in Day light	Colour under UV Lamp 254nm	Colour under UV Lamp 365nm	Colour in visible
Acetone	Dark green	Dark green	Orange	Dark green
Ethanol	Green	Green	Orange	Light green
Ethyl acetate	Dark green	Green	Orange	Light green
Chloroform	Green	Green	Orange	Puff colour
Petroleum ether	Brown	Light green	Brown	Brown
Water	Brown	Green	Honey colour	Brown

ii) Powder microscopy of the leaf of Cordia obliqua willd.

The powder microscopy of leaves shows the following characters.

- Lignified sclerenchyma cells
- Calcium oxalate crystals
- Epidermal cells with actinocytic stomata
- Trichome is nonglandular type bulbous basal part, sharp, thin pointed.
- Starch grains
- Fragments of spongy paranchyma cells.

Fig. 14 POWDER MICROSCOPY OF *Cordia obliqua willd*

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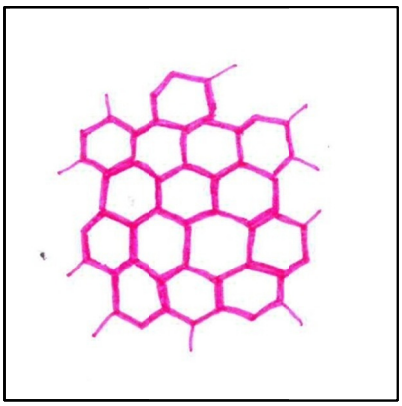

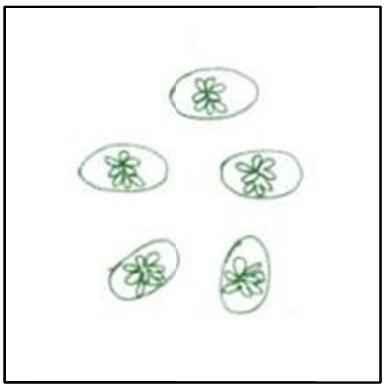

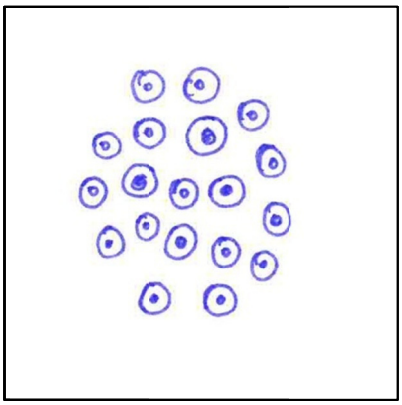

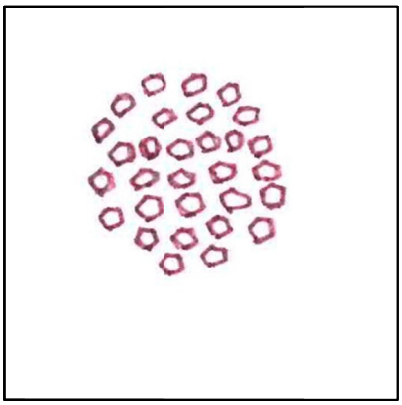

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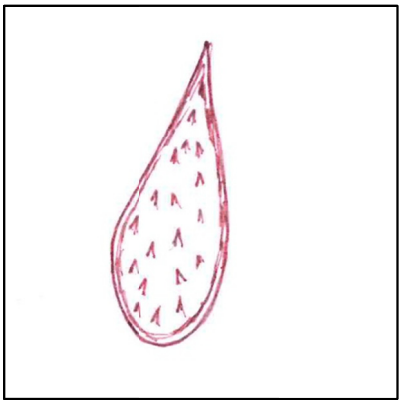
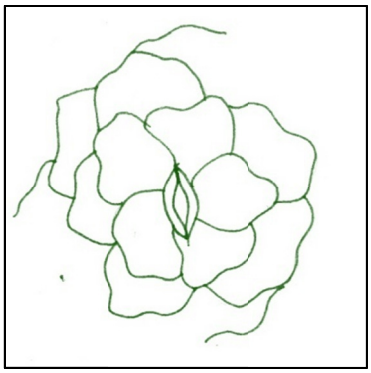




PHYTOCHEMICAL EVALUATION

CHAPTER –V

PHYTOCHEMICAL EVALUATION

SECTION – A

ORGANOLEPTIC EVALUATION

Nature – Coarse powder

Colour – Light Green colour

Odour– Characteristic odour

Taste – Characteristic taste

SECTION – B

PRELIMINARY PHYTOCHEMICAL SCREENING

Phytochemistry is widely used in the field of herbal medicine. Phytochemical characterization of plant material is important as it relates to the therapeutic actions. It is perhaps obvious that different species of plants would have different chemical constituents. However these differences can extent to different varieties or even the same varieties grow in different location or harvested at a different time, different parts of plant such as leaves, bark, seed, root, flowers and pods can also have different active constituents [(79)].

MATERIALS AND METHODS

QUALITATIVE CHEMICAL TESTS FOR THE LEAF POWDER AND CRUDE EXTRACTS [(80-82)]

Qualitative chemical tests are carried out for the purpose of specific identity of the substances in the crude extracts. The color reaction or precipitate usually observed by which we can identify a class of compound. Chemical tests can be useful for the investigation of the chemical compounds and to observe the efficiency of an extraction process. The petroleum ether, ether, ethyl acetate, methanol, ethanol and aqueous extracts were subjected to qualitative chemical tests. The various chemical tests performed on the extracts were for steroids, terpenoids, flavones, anthraquinones, sugars, glycosides, alkaloids, quinones, phenols, tannins and saponins and the results were recorded.

1**. Test for sterols**

The powdered leaf was first extracted with petroleum ether and evaporated to a residue. Then the residue was dissolved in chloroform and tested for sterols.

a.Salkowski's Test:

A few drops of concentrated sulphuric acid were added to the above solution, shaken well and set aside. The lower chloroform layer of the solution turns red indicating the presence of sterols.

b. Liebermann – Burchard's Test: To the chloroform solution a few drops of acetic anhydride and 1ml of concentrated sulphuric acid were added through the sides of the test tube and set aside for a while. At the junction of two layers a brown ring appears and the upper layer turned green indicating the presence of sterols.

2. Test for carbohydrates

a. Molisch's test:

To the aqueous extract and powdered drug of plants, add few drops of alcoholic – α – naphthol solution and sulphuric acid. A purple colour will be formed if carbohydrates are present.

b.Benedict's test:

The aqueous extract of the powdered leaf was treated with Benedict's reagent and boiled on a water bath and cooled. An reddish brown colour precipitate indicates the presence of carbohydrates.

b. Fehling's Test:

The aqueous extract of the powdered leaf was treated with Fehling's solution I and II and heated on a boiling water bath for half an hour. A red precipitate indicates the presence of free reducing sugars

3. Test for glycosides

a.Borntrager's Test.

The powdered leaf was boiled with dilute sulphuric acid, filtered and to the filtrate benzene was added and shaken well. The organic layer was separated, to which ammonia

solution was added slowly. No pink colour in the ammoniacal layer indicates the absence of anthraquinone glycosides.

Test for Cardiac Glycosides

a.KellerKiliani Test:

The powdered leaf was boiled with 10% alcohol for 2min, cooled and filtered. To the filtrate, lead sub acetate was added and filtered. The filtrate was then extracted with chloroform. The chloroform layer was separated and evaporated to dryness. The residue was dissolved in glacial acetic acid with traces of ferric chloride. To this few drops of sulphuricacid was added slowly along the sides of the test tube. No reddish brown ring at the junction and no green colour changes on standing indicates the absence of cardiac glycosides.

b.Legal test:

The powdered leaf was dissolved in pyridine, sodium nitroprusside solution was added to it and made alkaline. No pink or red colour indicates the absence of cardiac glycosides.

c.Baljet test:

To the powdered leaf sodium picrate solution was added. No yellow or orange colour indicates the absence of cardiac glycosides.

Test for coumarin glycosides [83]

A small amount powdered drug was placed in a test tube and covered with a filter paper moistured with sodium hydroxide solution. The covered test tube was placed on waterbath for several minutes. Then the paper was removed and exposed to UV light. No green colour fluorescence was observed indicating the absence of coumarin glycoside.

4. Test for Proteins and free amino acids

a.Millon'sTest :

A small quantity of aciduous – alcoholic extract of the powdered drug was heated with Millon's reagent. A white precipitate turning red on heating indicates the presence of proteins.

b.Biuret Test:

To the alcoholic extract of powdered drug, one ml of dilute sodium hydroxide(10%) solution was added followed by this one drop of very dilute copper sulphate solution was added. A violet colour indicates the presence of proteins.

5. Test for Mucilage

A few ml of aqueous extract was prepared from the powdered drug and treated with ruthenium red. A pinkish red colour indicates the presence of mucilage.

6. Test for Flavonoids

a.Shinoda test:

A little of the powdered drug was heated with alcohol and filtered. To the test solution magnesium turnings and few drops of concentrated hydrochloric acid were added and boiled for five minutes. Yellow colour indicates the presence of flavonoids.

b.Alkali Test:

To the small quantity of test solution 10% aqueous sodium hydroxide Solution was added. A yellow orange color indicates the presence of flavonoids.

c.Acid Test

To the alcoholic test solution, few drops of concentrated sulphuric acid was added. Yellow to orange colour was produced indicating the presence of flavonoids.

7.Test for terpenoids

The powdered leaf was shaken with petroleum ether and filtered. The filtrate was evaporated and the residue obtained was dissolved in small amount of chloroform and to the chloroform solution tin and thionyl chloride were added. No pink color indicates the absence of terpenoids.

8. Test for Tannins

A small quantity of the powdered drug was extracted with water. To the aqueous extract, few drops of ferric chloride solution was added. A bluish black color indicates the presence of tannins.

9. Test for Alkaloids

About 2gm of the powdered material was mixed with 1gm of calcium hydroxide and 5ml of water into a smooth paste and set aside for 5min. It was then evaporated to dryness in a porcelain dish on a water bath. To the residue, 20ml of chloroform was added, mixed well and refluxed for half an hour on a water bath. Then it was filtered and the chloroform was evaporated. To the residue, 5ml of dilute hydrochloric acid was added. The solution was divided into four parts and 2ml of each of the following reagents were added and the colour noted below indicates the presence of alkaloids.

a) Mayer's Reagent - Cream precipitate produced

b) Dragendorff's Reagent - Reddish brown precipitate produced

c) Hager's Reagent - Yellow precipitate

d) Wagner's Reagent - Reddish brown precipitate

Test for purine group (Murexide test)

The residue obtained after the evaporation of chloroform as described above was treated with 1ml of hydrochloric acid in a porcelain dish and 0.1gm of potassium chlorate

was added and evaporated to dryness on water bath. Then the residue was exposed to the vapour of dilute ammonia solution. No purple colour indicates the absence of purine group of alkaloids.

10.Test for saponin.

About 0.5 g of the powered material was boiled gently for 2 minutes with 20ml of water and filtered while hot and allowed to cool. 5 ml of the filterate was then diluted with water and shaken vigorously. The formation of frothing indicates the presence of saponin.

11. Test for volatile oil

About 100gm of fresh leaves were taken in a volatile oil estimation apparatus and subjected to hydro distillation for 4 hrs. No volatile oil was observed in the absence of volatile oil

The above chemical tests were carried out by using leaf powder and different plant extracts and the results were tabulated in **Tables 11&12** respectively**.**

Table 11: Preliminary phytochemical screening for the leaf powder of *Cordia obliqua willd.*

S.NO	TEST	RESULTS
1.	**TEST FOR STEROLS**	
	a. Salkowski's test	+
	b. Libermann- burchard's test	+
2.	**TEST FOR CARBOHYDRATES**	
	a. Benedict's test	+
	b. Fehling's test	+
3.	**TEST FOR PROTEINS**	
	a. Biuret test	+
	b. Xanthoprotic test	+
4.	**TEST FOR ALKALOIDS**	
	a. Mayer's reagent	+
	b. Dragendroff's reagent	+
	c. Hager's reagent	+
	d. Wagner's reagent	+
5.	**TEST FOR GLYCOSIDES**	
	a. Anthraquinone glycosides	
	i) Borntrager's test	-
	ii) Modified Borntrager's test	-
	b. Cardiac glycosides	
	i) Keller Killiani test	-
	ii) Baljet test	-
6.	**TEST FOR SAPONINS**	+
7.	**TEST FOR TANNINS**	
	a) $FeCl_3$ test	+
	b) Lead acetate test	+
8.	**TEST FOR FLAVONOIDS**	
	a. Shinoda test	+
	b.Alkali test	+
	c.Acid test	
9.	**TEST FOR TERPENOIDS**	-
10.	**TEST FOR VOLATILE OILS**	-
11.	**TEST FOR COUMARINS**	
	b) Fluorescence test	-

(+) indicates positive reaction (-) indicates negative reaction

Table: 12: Preliminary Phytochemical Screening for the various extracts of leaf powder of *Cordia obliqua*Willd.

Tests	Pet ether Extract	Methanol Extract	Ethanol Extract	Aqueous Extract
1.Test for sterols				
a.Salkowski's test	+	-	-	-
b.Libermann-Burchard's test	+	-	-	-
2. Test for carbohydrates				
a. Molisch's test	-	+	+	+
b. Fehling's test	-	+	+	+
c. Benedict's test	-	+	+	+
3. Test for proteins				
a. Millon's test	-	-	+	+
b.Biuret test	-	-	+	+
c. Ninhydrin test	-	-	+	+
4.Test for Alkaloids				
a.Mayer's reagent	-	-	+	-
b. Dragendroff'sreagent	-	-	+	-
c. Hager's reagent	-	-	+	-
d. Wagner's reagent	-	-	+	-
e. Test for purine group (Murexide test)	-	-	-	-
5. Test for Glycosides a.Anthraquinone glycosides				
i) Borntrager's test	-	-	-	-
ii) Modified Borntrager's test	-	-	-	-
b. Cardiac glycosides				
i) Keller Killiani test	-	-	-	-
ii) Baljet test	-	-	-	-
c.Cyanogenetic glycosides	-	-	-	-
d.Coumarin glycosides	-	-	-	-
6. Test for Saponins	-	-	+	+
7. Test for tannins				
i) $FeCl_3$ test	-	-	+	+
ii) Lead acetate test	-	-	+	+
8. Test for Flavanoids				
a. Shinoda test	-	+	+	+
b.Alkali test	-	+	+	+
c.Acid Test	-	+	+	+
9. Test for Terpenoids	-	-	-	--
10. Test for Volatile Oils	-	-	-	-
11. Test for Mucilage	-	-	-	+

(+) indicates positive reaction (-) indicates negative reaction

The ethanolic extract of *Cordia obliqua* showed the presence of alkaloids, flavones, carbohydrates, phenols, tannins, saponins and proteins. The petroleum ether extract showed the presence of sterols. None of the extracts showed the presence of anthraquinones and terpenoids.

SECTION -C

QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

The medicinal value of the plants lies in the bioactive phytochemical constituents that produce definite physiological effect on human body [84] These natural compound formed the base of modern drugs as we use today [85]. Phytoconstituents are the natural bioactive compounds found in plants. These phytoconstituents work with nutrients and fibres to form an integrated part of defense system against various diseases and stress conditions [86]. The most important of these bioactive constituents of plants are tannins, flavonoids, Phenolic compounds[87,88]. Flavonoids, a group of polyphenolic compounds with known properties such as free radical scavenging activity, inhibition of hydrolytic and oxidative enzyme and anti- inflammatory action [89,90].

1. Determination of total phenolic content [91-93]

Principle

The total phenol content of *Cordia obliqua*, was determined by the Folin-Ciocalteu colorimetric method [91]**.** The FolinCiocalteu reagent is a mixture of phosphomolybdate and phosphotungstate. The method measures the amount of substance needed to inhibit the oxidation of the reagent **[92].**

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

10% sodium carbonate

1N Folin-Ciocalteu reagent (Reagent diluted with equal volume of water)

Procedure

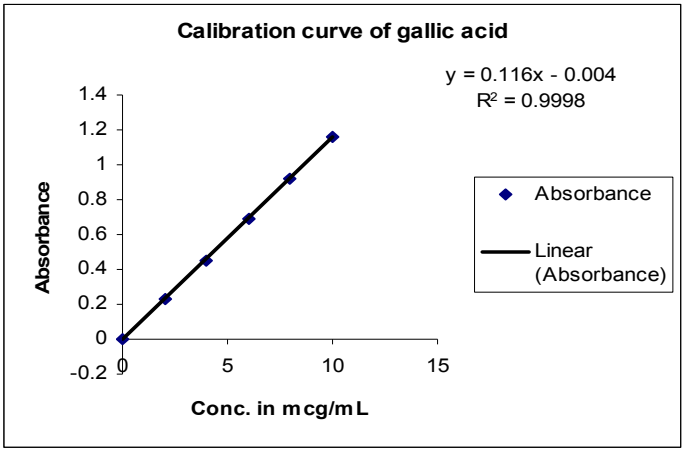
The compound gallic acid was used as standard [93] and was weighed and dissolved in distilled water to produce 1mg/ml stock solution. The stock solution was further diluted to get concentrations ranging from 2-10µg/ml. To these solutions, 0.5ml of Folin-Ciocalteu reagent and 0.5ml of sodium carbonate was added and the final volume was made up to 10ml with distilled water. The absorbance was measured at 760nm after incubation at room temperature for 30min. The ethanolic extract (0.5ml & 1ml of 1mg/ml) was mixed with 0.5ml of Folin-Ciocalteu reagent and 0.5ml of 10% sodium carbonate and final volume was made up to 10ml with distilled water, and the absorbance was measured at 760nm after incubation at room temperature for 30min. A calibration curve was constructed by plotting concentration versus absorbance of gallic acid (**Fig.15).** A linear regression equation was formed and the amount of phenolic compounds was determined by using the equation. The total phenolic content was expressed as mg gallic acid equivalents (GAE)/g of extract. The results were tabulated in **Table 13.**

Table: 13: Estimation of total phenolic content of ethanolic extract of *Cordia obliqua willd.*

S. No.	Conc. of gallic acid in µg/ml	Absorbance at 760nm	Conc. of ethanolic extract in µg/ml	Absorbance at 760nm*	Amount of total phenolic content in terms mgGAE/g of extract*
1	2	0.229 ± 0.010	50	0.310±0.00	54.24± 0.54
2	4	0.452 ± 0.006	100	10.746±0.01	64.70± 1.50
3	6	0.695 ± 0.005		**Average**	**59.47 ± 1.02**
4	8	0.918 ± 0.031			
5	10	1.162 ± 0.028			

*** mean of three readings ±SEM**

Fig:15: Calibration curve of gallic acid



The linear regression equation was found to be y = 0.116x-0.004 while the correlation was found to be 0.9998. The amount of phenolic content present in the extract in terms of mg GAE/g of extract was found to be 59.47 ±1.02 by using the above linear regression equation.

Determination of total flavonoid content [94-95]

Principle

The aluminum chloride colorimetric technique was used for the estimation of total flavonoid estimation [94]. Aluminum ions form stable complexes with C4 keto group and either C3 or C5 hydroxyl groups of flavones and flavonols in acidic medium. It also forms acid labile complexes with ortho hydroxyl groups in the A or B rings of flavonoids [95]. These complexes showed a strong absorption at 415nm which is used for the estimation of flavonoids.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

10% aluminum chloride

1M potassium acetate

Procedure

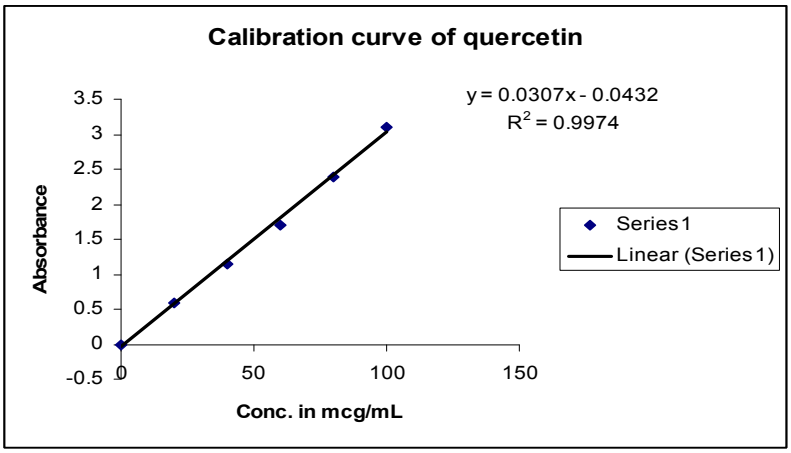
A known quantity of quercetin [96]was dissolved in ethanol to get a stock solution of 1mg/ml. Further dilutions were made to get concentrations ranging from 20-100µg/ml. 1ml of the above standard solutions were taken in different volumetric flasks, 0.1ml of aluminum chloride solution, 0.1ml of potassium acetate solution and 2.8ml of ethanol were added and the final volume was then made up to 5ml with distilled water. After 20min the absorbance was measured at 415nm. A sample without aluminium chloride was used as a blank. From the absorbance obtained, a calibration curve was constructed by plotting concentration versus absorbance of quercetin (**Fig.16**). 0.5ml and 1ml of ethanolic extract of *Cordia obliqua* at a concentration of 1mg/ml were taken and the reaction was carried out as above and the absorbance was measured at 415nm after 20min and the readings were tabulated in **Table14.** The amount of flavonoids present can be determined by linear regression analysis. The total flavonoid content was expressed as mg querecetin equivalents /g of extract.

Table: 14: Estimation of total flavonoid content of ethanolic extract of *Cordia obliqua*

S. No.	Conc. of quercetin in µg/mL	Absorbance at 415nm	Conc. of ethanolic extract in µg/ml	Absorbance at 415nm	Amt of total flavonoid content in terms mg quercetin equivalent/ g of extract
1	20	0.589 ± 0.01	100	0.196 ± 0.00	162.99±0.75
2	40	1.151 ± 0.04	200	0.403± 0.00	144.90±1.66
3	60	1.710 ± 0.09		**Average**	**153.94±1.20**
4	80	2.390 ± 0.03			
5	100	3.112 ± 0.03			

***mean of three readings ± SEM**

Fig.16 : Calibration curve of quercetin



The linear regression equation was found to be y = 0.0307x-0.0432, while the correlation was found to be 0.9974. The amount of flavonoid content present in the ethanolic extract of *Cordia obliqua* in terms mg quercetin equivalent/g of extract was found to be 153.94 ± 1.20 mg/g of extract by using the above linear regression equation.

Determination of total tannin content [96-98]

Principle

Total tannin content of ethanolic extract of *Cordia obliqua* was determined by Folin Denis reagent method [97]. Tannin like compounds reduces phosphotungstomolybdic acid in alkaline solution to produce a highly blue coloured solution [98]. The intensity is measured spectrophotometerically at 700nm

Reagents

a) Folin Denis Reagent (sodium tungstate 100g and phosphomolybdic acid 20gm were dissolved in distilled water 750ml along with phosphoric acid 50ml. The mixture was refluxed for 2 hrs and the volume was madeupto 1 litre with distilled water)
b) Sodium carbonate solution (10%)

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Procedure

0.1ml,0.2ml& 0.4ml of (1mg/ml) ethanolic extracts of *Cordia obliqua* was taken in a separate test tubes and mixed with distilled water to made upto the volume of 1ml. Then add 0.5ml of Folin Denis reagent and allowed to stand for 15 mins, then 1ml of 10% sodiumcarbonate solution was added and the mixture was mixed with distilled water and made upto 10ml, allowed to stand for 30mins at room temperature and the tannin content was determined spectrophotometrically at 700nm.

The calibration curve was generated by preparing tannic acid at different concentration (4, 8, 12, 16, 20µg/ml). The reaction mixture without sample was used as

blank. The total tannin content in the ethanolic extract of *Cordia obliqua* was expressed as milligrams of tannic acid equivalent per gm of extract.

Total tannin content

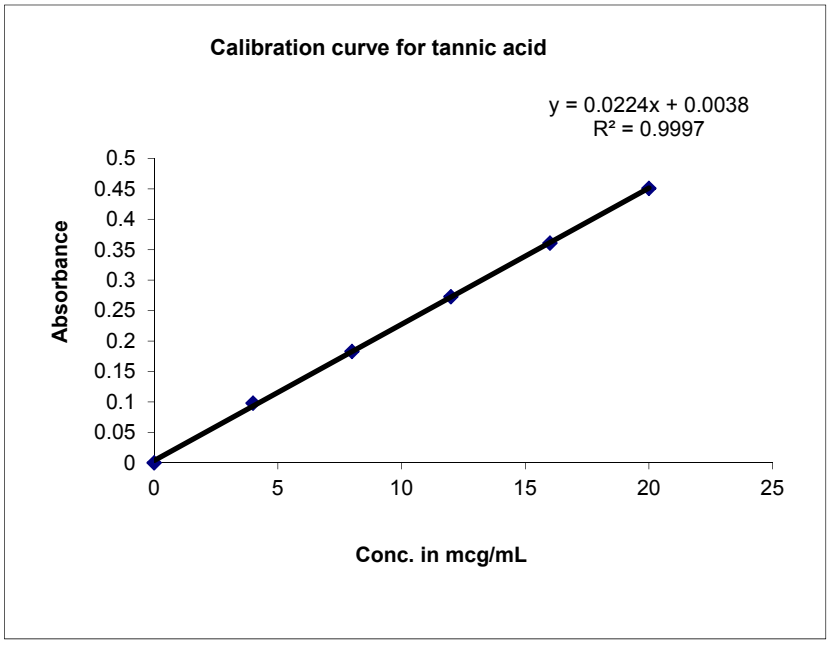
The results for total flavonoid content of 70% ethanolic extract of *Cordia obliqua* were presented in **Table15.**

Table: 15: Estimation of total tannins content of ethanolic extract of *Cordia obliqua*

S. No.	Conc. of quercetin in µg/mL	Absorbance at 415nm	Conc. of ethanolic extract in µg/ml	Absorbance at 700nm	Amt of total tannin content in terms mg tannic acid equivalent/ g of extract
1	40	0.098±0.02	10	0.031±.00	125.1±1.50
2	80	0.183±0.01	20	0.079±0.00	172.4±0.75
3	120	0.203±0.01	40	**0.179±0.00**	199.8±0.99
4	160	0.361±0.2		**Average**	**165.76±1.08**
5	200	0.451±0.1			

***mean of three readings ± SEM**

Fig .17 :calibration curve for tannic acid



The linear regression equation was found to be y = 0.0224x+ 0.0038 while the correlation was found to be 0.9974. The amount of flavonoid content present in the ethanolic extract of *Cordia obliqua* in terms of mg quercetin equivalent/g of extract was found to be165.7±1.08mg/g of extract by using the above linear regression equation

SECTION -D

CHROMATOGRAPHY

Chromatography is the separation of the compounds of mixtures by their continuous distribution between two phases. The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile phase and stationary phase [(99)].

The various types of chromatography include paper chromatography (PC), thin layer chromatography (TLC), column chromatography (CC), gas chromatography (GC), high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC).

Thin Layer Chromatography [(100,101)]

Thin layer chromatography (TLC) is a chromatographic technique used for the separation of mixtures. The principle involved is adsorption. The solute competes with the solvent for the surface sites on the adsorption. Depending on the distribution coefficients, the compounds are distributed on the surface of the adsorbant.The compounds, which is readily soluble but not strongly adsorbed, moves up along with the solvent and that compounds not

so soluble but more strongly adsorbed move up less readily leading to the separation of compounds.

Preparation of TLC Plates

The adsorbent (silica gel G) slurry was prepared in water in the ratio of (1: 2). The glass plates (20cm x 5cm) were cleaned and laid in a row as a template, the suspension was poured into Stahl TLC spreader, which was adjusted to 0.25mm thickness and coated in a single passage of the spreader over them. These plates were air dried and activated in hot air oven at 105ºC for 30min and kept in a dessicator. The plates were used as the stationary phase or Pre-coated aluminum plates coated with silica gel G F_{254} (Merck) were also used for analysis.

Sample application

The sample was prepared by dissolving the ethanolic extract of *Cordia obliqua* in ethanol to get 5mg/ml. The sample was applied as a spot with the help of capillary tube.

Development of the chromatogram

The extracts were dissolved in ethanol and the spot was applied on the TLC plates using capillary tube.

The plates were developed in the chromatographic tank containing the solvent systems. Various solvent systems were tried for better results. The TLC plates were allowed to develop upto 2/3rd of the plate length and dried. The TLC plates were examined visually or under UV light.

Stationary phase - silicagel G

Mobile phase - Ethylacetate:Benzene(2:8)

- Chloroform: Ethylacetate(6:4)

Detecting agent - Visual & UV light

The Rf value of the spots obtained were calculated by using the formula,

$$R_f = \frac{\text{Distance Travelled by Solute}}{\text{Distance Travelled by Solvent}}$$

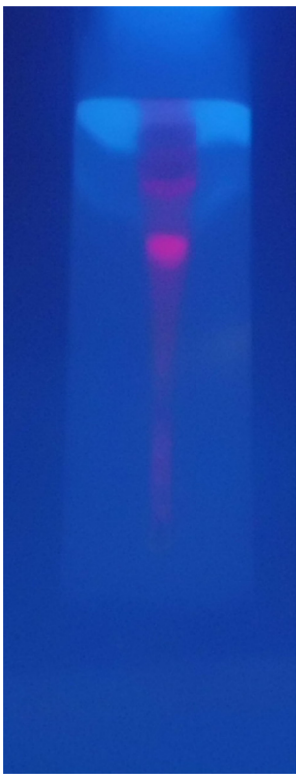
Two spots showed fluorescence when viewed under UV light. The R_f value of 0.35, 0.78 at 365nm for the ethanolic extract of *Cordia obliqua leaves* in Ethyl acetate: Benzene (2:8). The extract also showed 2fluorescent spots at R_f value of 0.52 and 0.98 when viewed under UV at 365nm after development in the mobile Chloroform:ethyl acetate(6:4).

Table16: TLC of the Ethanolic extract of *Cordia obliqua willd*

S. No	**Solvent system**	**Detecting agent**	**No of spots**	**Colour of spots**	**$R_f$values**
1.	Ethyl acetate: Benzene (2:8)	Under UVlight at 365nm	I	Yellow colour	0.35
			II	Orange fluorescence	0.78
2.	Chlopoform: ethyl acetate (6:4)	Under UVlight at 365nm	I	Yellow colour	0.52
			II	Orange fluorescemce	0.98

Fig.18: TLC of ethanolic extract of *Cordia obliqua willd.*

Solvent system-I **Solvent system-II**



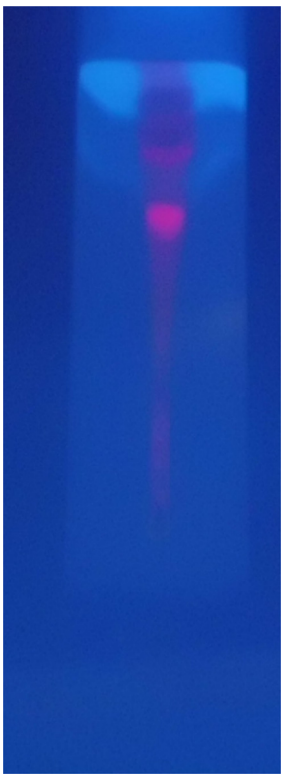


Fig. 19: Visualization at 254nm and 366nm and white light

White light **@ 254nm** **@366nm**

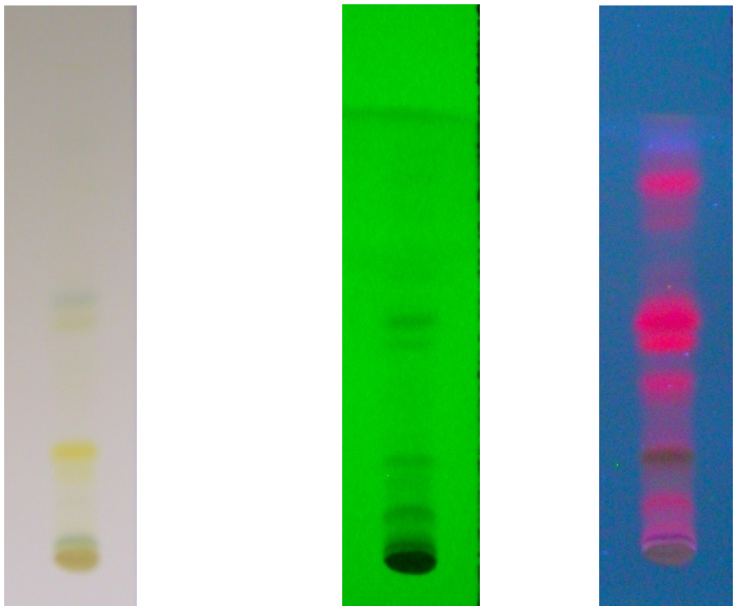
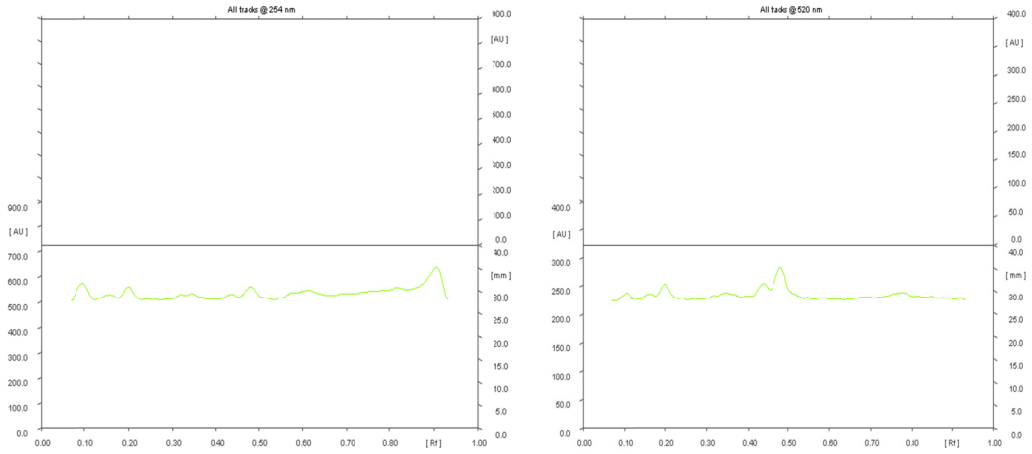


Fig. 20: 3D Display of the fingerprint profile at 254nm and 366nm

White light **@ 254nm**



@ 366nm

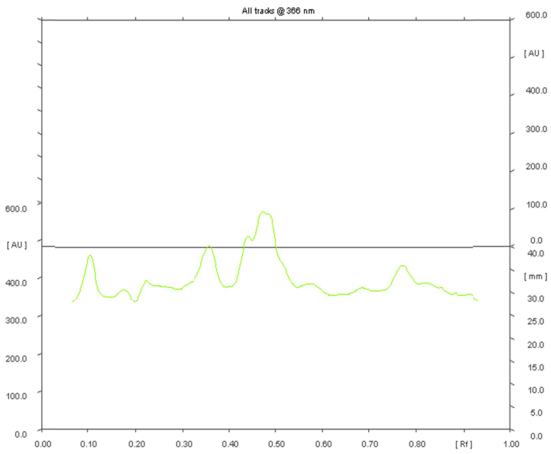
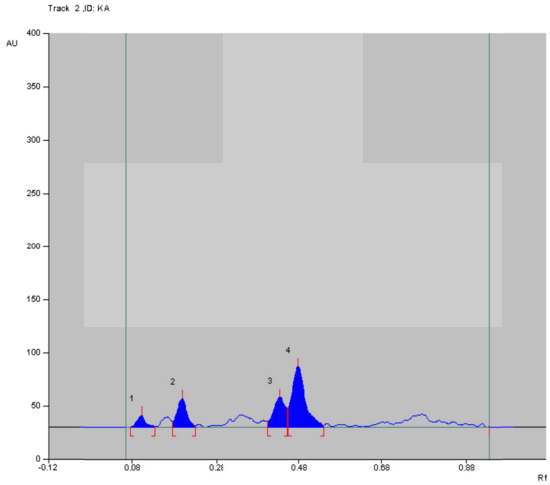
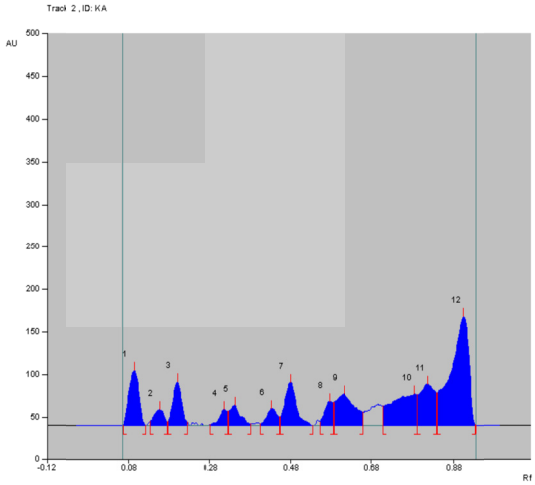


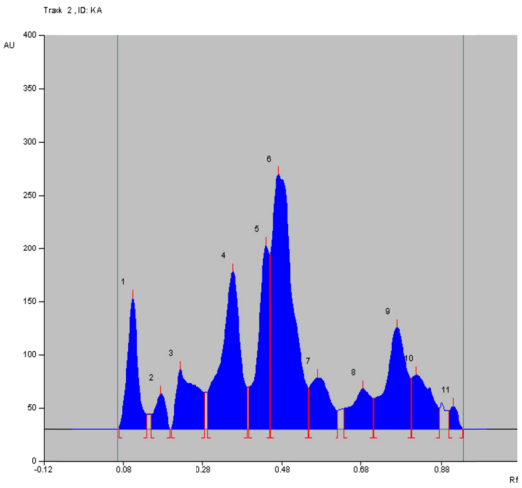
Fig. 21: Peak display of ethanolic extract of*Cordia obliqua* at 254nm and 366nm

White light **@ 254nm**





@ 366nm



SECTION -E

HIGH PERFOMANCE THIN LAYER CHROMATOGRAPHY ANALYSIS OF 70% ETHANOLIC EXTRACT OF *CORDIA OBLIQUA* WILLD. LEAVES

High performance thin layer chromatography (HPTLC)is a modern adaptation of TLC with improved versatility, separation efficacy and detection limits. HPTLC is useful for the identification of plants and their extracts because each plant species produces a distinct chromatogram, with unique marker compounds used for plant identification.

This is a sophisticated advancement in thin layer chromatography. It has advantages like better resolution, faster development of spots and also easy detection and quantification of separated compounds.

APPLICATION OF HPTLC

1. For the detection and analysis of components in phytochemistry, medicinal chemistry and organic chemistry.
2. Compounds which are very complex or those in very scarce quantities can be analyzed [(102)].

HIGH PERFOMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

Instrument used **:** CAMAG make HPTLC.

Software : winCATS 1.4.3

Sample Applicator : Linomat 5.

Detection : @520nm, @254nm & @366nm in Densitometry TLC Scanner 3

Sample preparation : sample was dissolved in 1 ml ethanol

Stationary Phase : HPTLC plates silica gel 60 F 254.

Mobile Phase **:** Ethyl acetate: Toluene (2:8)

Sample Solution **:** 2µl sample is applied as 8mm band.

PROCEDURE

The samples (10µl) were applied as spot, about 6mm from the edge of the TLC plates. The plates were developed upto 120mm in the mobile phase.

Table: 17: HPTLC profile of ethanolic extract of *Cordia obliqua willd* leaves

S.No	White light		@ 254nm		@ 366nm	
	Rf value	**Area (AU)**	**Rf value**	**Area (AU)**	**Rf value**	**Area (AU)**
1	0.10	174.9	0.10	1195.1	0.10	2425.5
2	0.20	485.6	0.16	355.9	0.17	658.3
3	0.44	602.3	0.20	834.8	0.22	2266.9
4	0.48	1396.3	0.32	276.3	0.36	5366.4
5			0.34	479.9	0.44	3785.8
6			0.43	383.0	0.47	9496.0
7			0.48	1084.3	0.57	1853.3
8			0.58	447.8	0.69	1385.3
9			0.61	1390.7	0.77	3641.1
10			0.79	1695.7	0.82	1827.4
11			0.82	1358.7	0.91	324.2
12			0.91	4310.4		

PHARMACOLOGICAL SCREENING

CHAPTER – VI

PHARMACOLOGICAL SCREENING

SECTION - A

IN-VITRO ANTIOXIDANT ACTIVITY

Antioxidants regarded as compounds which are able to delay, retard or prevent oxidation process[103]. Reactive oxygen species (ROS) are generated in the normal metabolism of living organisms and besides of their useful role in signal transduction, they are also involved in the dispersion of several degenerative disease like malignant tumors,rheumatic joint inflammation, cataracts, Parkinson's and Alzheimer's disease, hypertension, diabetes, oxidative stress, tissue damage and atherosclerosis.[104]. The role of medicinal plants in disease prevention or control has been attributed to the antioxidant properties of their constituents such as vitamins, terpenoids, phenolic acids, stilbenes, tannins, flavonoids,quinones, coumarians, alkaloids, which are rich in antioxidant activity[105]. The ingestion of natural antioxidants has been associated with reduced risk of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing [106,107].

Method 1: Free radical Scavenging activity using diphenylpicrylhydrazyl (DPPH) free radical

The free radical scavenging activity of the extracts is evaluated by assessing their ability to reduce the colour of DPPH in ethanol according to Brand Williams [108]. DPPH is a stable, free radical and this method is an easy, rapid and sensitive way to survey the antioxidant activity of specific compound or plant extracts. [109]

Principle

This is a simple method that has been developed to determine the antioxidant activity of plants which utilizes the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The odd electron in the DPPH free radical gives a strong absorption maximum at 517nm and is purple

in color. The color turns from purple to yellow as the molar absorptivity of the DPPH radical at 517nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to number of electrons captured [110].

$$DPPH^{\cdot} + AH \rightarrow DPPH - H + A^{\cdot}$$

Instrument

Shimadzu UV Visible spectrometer, Model 1800

Reagents

0.1mM Diphenyl Picryl Hydrazyl (DPPH) in ethanol.

Procedure[109,110,)]

A stock solution of DPPH was prepared in ethanol(4mg/100ml). 1mg/1ml concentration of ethanolic extract of *Cordia obliqua leaves* was prepared. To 1 ml of the various concentrations of test samples(0.5ml,0.6ml,0.7ml and 0.8ml of extract in ethanol), 4ml of DPPH solution was added. Control was prepared without sample in an identical manner. Ethanol was used as blank. The reaction was allowed to be complete in the dark for about 30min. Then the absorbance was measured at 517nm. Vitamin C was used as standard. The percentage scavenging was calculated using the formula [(Control-Test)/Control] x 100. A graph was constructed by plotting concentration versus percentage inhibition. The concentration of the sample required for 50% reduction in absorbance (IC_{50}) was calculated using linear regression analysis. A triplicate reading was taken and the average was calculated. The results obtained were presented in **Table- 18**and **Fig. 22.**

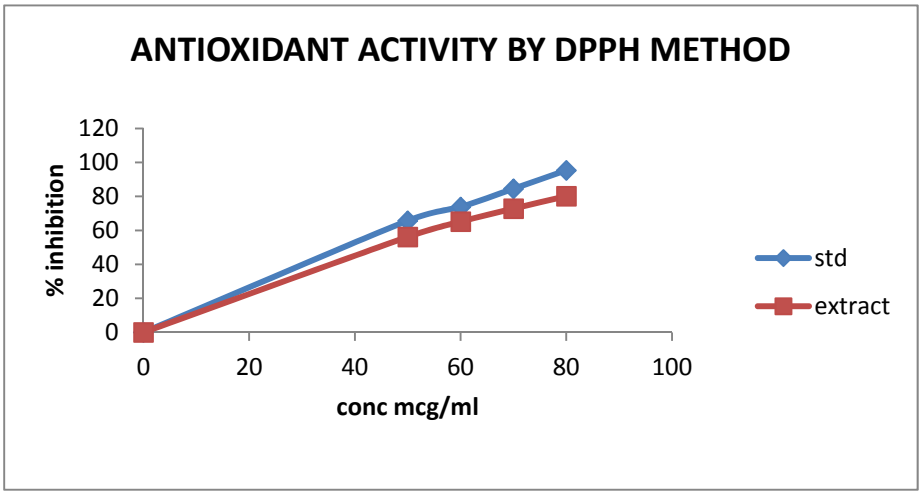
Method I: Free radical Scavenging activity using diphenylpicrylhydrazyl (DPPH) free radical

Table 18: Percentage inhibition of ethanolic extract of *Cordia obliqua leaves* and standard ascorbic acid against DPPH at 517nm

S. No.	Conc. in µg/mL	Percentage inhibition by ascorbic acid	Percentage inhibition by *Extract*
1	50	65.72±3.18	56.2±1.34
2	60	73.8±2.85	65.16±1.30
3	70	84.49±1.55	72.85±1.50
4	80	95.39±1.28	80.16±1.44
	IC_{50}	**40.42µg/ml**	**47.23µg/ml**

***mean of three readings ± SEM**

Fig. 22 – Free radical scavenging activity of ethanolic extract of *Cordia obliqua leaves (EECO)* by DPPH method at 517nm



From the results, obtained it has been observed that, the ethanolic extract of *Cordia obliqua* showed a percentage inhibition of 80.16±1.44 while ascorbic acid showed a percentage inhibition of 95.39± 1.28 at a concentration of 80µg/ml. The IC_{50} value calculated using the linear regression analysis was found to be 47.23 and 40.42ssµg/ml for the ethanolic extract and ascorbic acid respectively. This study showed that the extract possess a significant free radical scavenging capacity.

Method 2: Ferric Reducing Antioxidant Power (FRAP) Assay [111,112]

Total antioxidant activity is measured by FRAP assay of Benzie *et al.,* (1999) [111]. The ferric reducing antioxidant power assay measures the potential of antioxidants to reduce the Fe^{3+} and 2,4,6 tripyridyl-s-triazyne (TPTZ) complex present in stoichiometric excess to the blue coloured Fe^{2+} complex which increases the absorption at 593nm.

Principle

At low pH, reduction of ferric tripyridyltriazine (Fe III TPTZ) complex to ferrous form(which has an intense blue colour) can be monitored by measuring the change inabsorption at 593nm. The reaction is non-specific, in that any half reaction that haslower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferric to ferrous (Fe III to Fe II) ion formation. The change in absorbance istherefore, directly related to the combined reducing power of the electron donating antioxidants present in the reaction mixture.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

FRAP Reagent

a) Acetate buffer 30mM pH 3.6: Weigh 3.1g sodium acetate trihydrate and add 16 ml of glacial acetic acid and make the volume to 1 L with distilled water.

b) TPTZ (2, 4, 6-tripyridyl-s- triazine) (M.Wt. 312.34) 10mM in 40mM HCl

c) $FeCl_3$. $6H_2O$ (M.Wt. 270.30) 20mM

The FRAP reagent was prepared freshly by mixing a,b& c in the ratio of 10:1:1 at the time of use.

Procedure

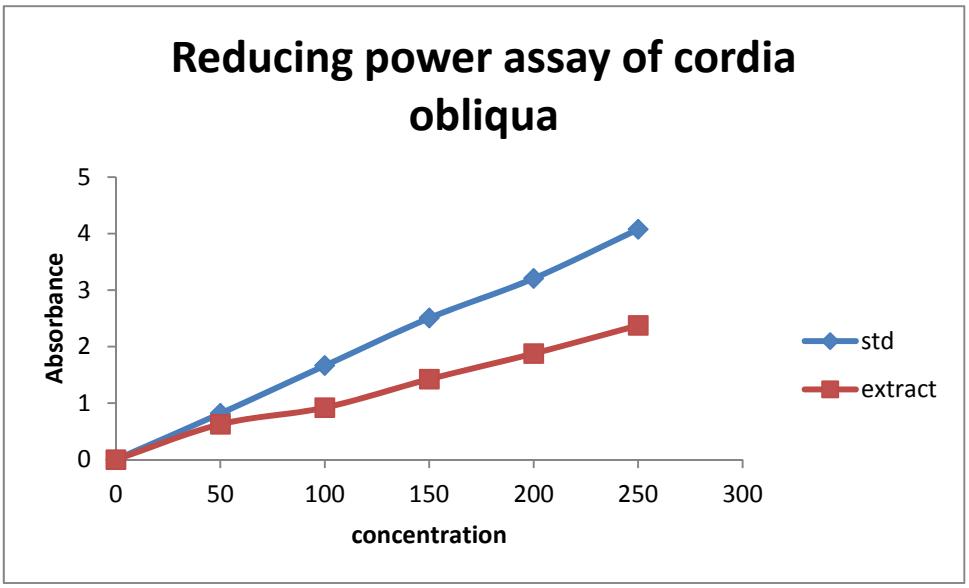
50µl, 100µl, 150µl, 200µl and 250µl of 2mg/ml concentration of ethanolic extract of *Cordia obliqua* were taken and mixed with 3ml of working FRAP reagent and absorbance was measured at 0min at 593nm. Thereafter samples were placed at 37ºC in water bath and absorption was again measured after 4min. Ascorbic acid was used as standard. The FRAP value of the sample was calculated using this equation:[Change in absorbance of sample from 0-4min/ change in absorbance of standard from 0-4min] * Frap value of standard was also observed.The results obtained for the FRAP assay were presented in the **Table. 19** and **Fig. 23.**

Table:19: Ferric reducing antioxidant assay of ascorbic acid andethanolic extract of *Cordia obliqua leaves*

S. No	Conc. in µg/ml	Absorbance of ascorbic acid	Absorbance of ethanolic extract
1	50	0.817±0.06	0.625±0.02
2	100	1.663±0.05	0.920±0.02
3	150	2.504±0.05	1.424±0.01
4	200	3.206±0.03	1.877±0.15
5	250	4.075±0.06	2.374±0.231

***Mean of three readings ± SEM**

Fig. 23: Ferric reducing anti-oxidant assay of ethanolic extract of *Cordia oblique leaves*



From the table, it can be seen that the ethanolic extract of *Cordia obliqua* leaves showed an absorbance of 2.374 ±0.231 for a concentration of 250µg/mlwhile ascorbic acid showed an absorbance of 4.075 ±0.06 at a concentration of 250µg/ml. The extract shows a dose dependent reducing ability. The graphical representations of the reducing power activity of the ethanolic extract of *Cordia obliqua* and ascorbic acid were presented in **Fig 23.**

Method 3:Total antioxidant activity by Phosphomolybdenum assay[113,114]

Principle

Total antioxidant capacity was measured by spectrophotometric method of Prieto*et al*(1999)[113]. Phosphomolybdenum method assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate Mo (V) complex at acidic pH and the absorbance was measured at 695nm. This method is used to determine the total antioxidant activity of samples.

$Mo^{6+} \rightarrow Mo^{5+}$

Reagents

0.6M sulphuric acid

28mM sodium phosphate

4mM ammonium molybdate

Instruments

Shimadzu UV Visible spectrophotometer, Model 1800

Procedure

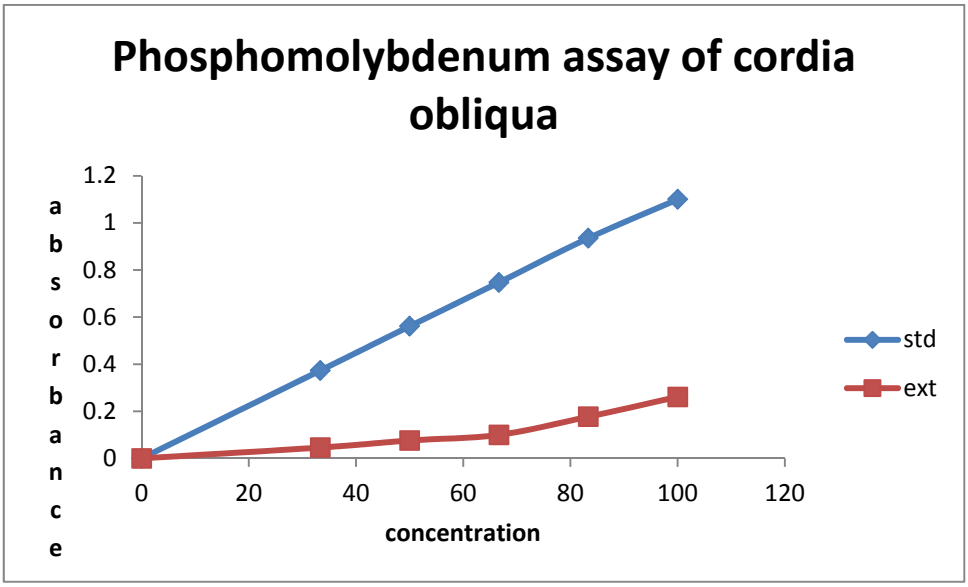
An aliquot of 0.3ml of different concentrations of sample solutions was combined with 2.7ml of the reagent solution (H_2SO_4, sodium phosphate and ammonium molybdate). In case of blank, 0.3ml of ethanol was used in place of sample. The tubes were incubated for 95°C for 90min. After the mixture was cooled to room temperature, the absorbance was measured at 695nm against blank. The standard Ascorbic acid was treated in a similar manner. The total antioxidant activity is expressed as the number of equivalents of ascorbic acid[114].

Table 20: Percentage inhibition of ascorbic acid and ethanolic extract of *Cordia obliqua* by Phosphomolybdenum method at 695nm

S. No.	Conc. in µg/ml	Percentage inhibition by standard ascorbic acid	Percentage inhibition by extract
1	33.33	0.373±0.03	0.046± 0.01
2	50	0.561 ± 0.01	0.076± 0.01
3	66.66	0.747± 0.00	0.100 ± 0.00
4	83.33	0.935 ± 0.01	0.177 ± 0.01
5	100	1.1±1.00	0.261±0.01

***mean of three readings ± SEM**

Fig 24 : Percentage inhibition of ascorbic acid and ethanolic extract of *Cordia obliqua* byPhosphomolybdenum method at 695nm



From the table, it can be seen that the ethanolic extract of *C. obliqua* showed an absorbance of 0.261± 0.01 for a concentration of 100µg/mlwhile ascorbic acid showed an absorbance of 1.1±0.02 at a concentration of 100µg/ml. The extract shows a dose dependent reducing ability. The graphical representations of the reducing power activity of the ethanolic extract of *Cordia obliqua* and ascorbic acid are presented in **Fig 24.**

Method 4: Nitric oxide scavenging activity assay [115,116]

Principle

Nitric oxide scavenging activity was determined according to the method reported by Green *et al*., (1982) [115]. The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. The nitrite ions produced diazotizes sulphanilamide and then the diazonium salt reacts with N,N naphthyl ethylene diaminedihydrochloride to give a pink colourchromophore which has a maximum absorption at 546nm.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

10mM sodium nitroprusside

Phosphate buffered saline pH 7.4

2% sulphanilamide in ortho phosphoric acid

0.1% naphthyl ethylene diaminedihydrochloride

Procedure

To 1ml of freshly prepared solution of sodium nitroprusside, 2.5ml phosphate buffered saline pH 7.4 was added and mixed with 1ml of extracts at various concentrations (0.2,0.4,0.6,0.8 and 1ml of 1mg/ml), then the mixture was incubated at 25^{o}C for 30min. From the incubated mixture 1.5ml was taken. To this, 1ml of sulphanilamide in phosphoric acid and 0.5ml of naphthyl ethylene diaminedihydrochloride were added and the absorbance was measured at 546nm using reagent as blank. A control without the extract was also prepared and subjected to the above procedure. Ascorbic acid was used as a standard.

The percentage inhibition of nitric oxide radical generated was calculated using the following formula: % inhibition = [(Control-Test)/Control] x 100.

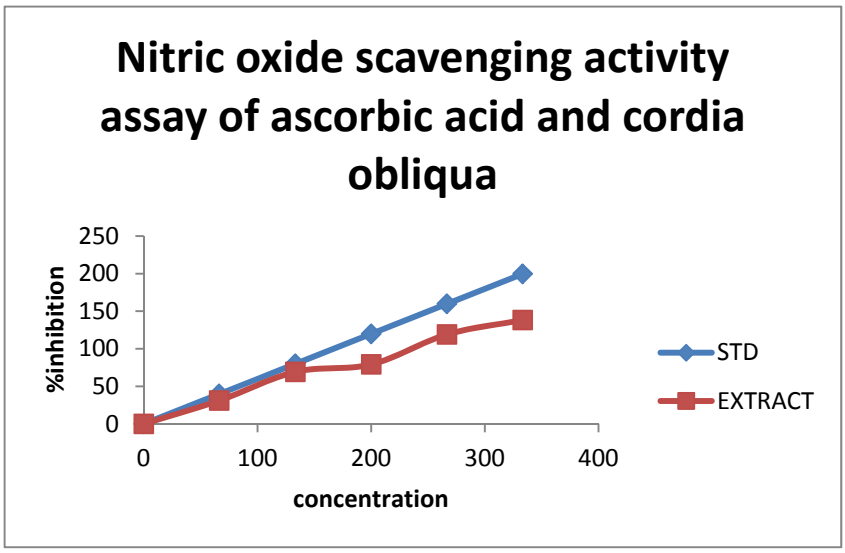
A graph was constructed using concentration versus percentage inhibition and the linear regression equation calculated. The IC_{50} was calculated using linear regression analysis. The results obtained for the nitric oxide scavenging activity assay were presented in **Table 21** and **Fig 25.**

Table 21: Percentage inhibition of ascorbic acid and ethanolic extract of *C.obliqua* against nitric oxide at 546nm

S. No.	Conc. in µg/mL	Percentage inhibition by standard ascorbic acid	Percentage inhibition by extract
1	66.33	39.73±0.21	31.07 ± 1.38
2	133.33	79.87± 0.44	69.27 ± 1.03
3	200	119.8 ± 0.66	79.13 ± 1.10
4	266.66	159.74 ± 0.80	119.05 ± 2.81
5	333.33	199.67±1.10	138.19±1.18
	IC_{50}	**83.47µg/ml**	**111.47µg/ml**

***mean of three readings ± SEM**

Fig 25: Nitric oxide scavenging activity of ascorbic and ethanolic Extract of *C.obliqua*



From the table, it can been seen that the ethanolic extract of *Cordia obliqua leaves* showed a percentage inhibition of 138.19 ± 1.18 while ascorbic acid showed a percentage inhibition of 199.67 ± 1.10 at a concentration of 333.33µg/mL. The IC_{50} value calculated using the linear regression analysis was found to be 111.47and 83.47µg/mL for ethanolic extract and ascorbic acid respectively.

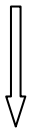
Method 5 : Reducing power assay [117,118]

Principle

Reducing power assay is a spectrophotometric method and is based on the principle that increases in absorbance of the reaction mixture indicates the increases in the reducing power of the sample.Antioxidant activity may be due to a variety of mechanism viz.,the prevention of chain initiation , the binding of transition metal ion catalysts, decomposition of peroxides,thereductive capacity and free radical scavenging. The assay is based on the reduction of ferric in potassium ferricyanide to ferrous to form potassium ferrocyanide by the

sample and the subsequent formation of Prussian blue colour with ferric chloride. The absorbance of the blue complex is measured at 700nm.

Potassium ferricyanide+ Ferric chloride



Antioxidant

Potassium ferrocyanide + Ferrous chloride

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Materials required

70% Ethanolic extract of leaves of *Cordia obliqua*

Ascorbic acid

1% w/v Potassium ferricyanide

10% w/v Trichloroacetic acid

0.2M, Phosphate buffer (pH 6.6)

0.1% w/v Ferric chloride

Procedure

The reducing power ability of plant extracts was screened by assessing the ability of the test extract to reduce $FeCl_3$solution as mentioned by Oyaizu etal (1986) [(118)]. 0.1 to 0.5 ml of plant extract solution (1mg/ml) was mixed with 0.75ml phosphate buffer and 0.75ml of 1% potassium ferricyanide $[K_3Fe(CN_6)]$ and incubated at 50^oC for 20min.0.75ml of 1%trichloroacetic acid was added to the mixture ,allowed to stand for 10min. The whole mixture was then centrifuged at 3000rpm for 10min. Finally 1.5ml of the supernatant was removed and mixed with 1.5ml of distilled water and 0.1ml of 0.1%ferric chloride solution and the absorbance measured at 700nm in UV-Visible Spectrophotometer. Ascorbic acid

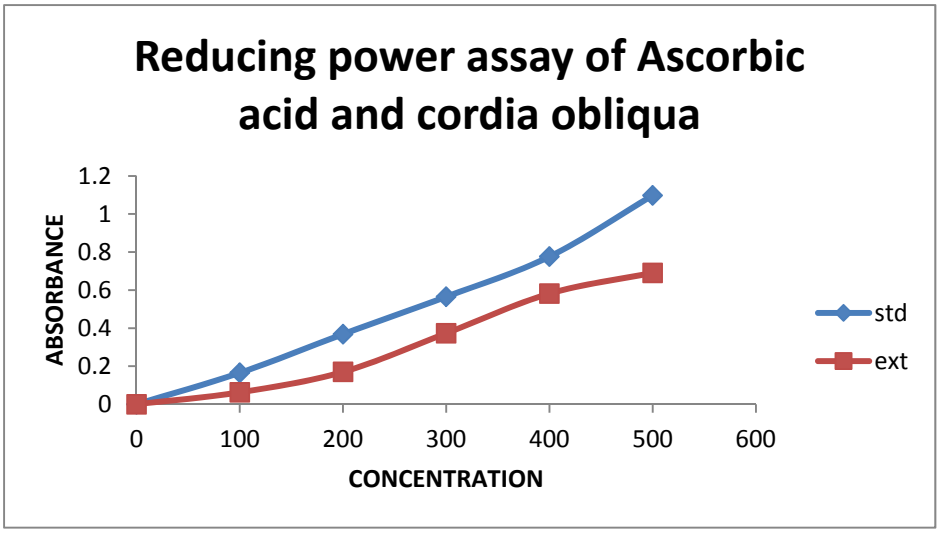
was used as standard and phosphate buffer used as blank solution. The results obtained for the free radical scavenging activity against reducing power assay were presented in **Table 22.**

Table 22: Reducing power assay of ascorbic acid and ethanolic extract of *Cordia obliqua leaves*

S. No.	Conc. in µg/ml	Percentage inhibition by standard ascorbic acid	Percentage inhibition by extract
1	100	0.165±0.007	0.062 ± 0.001
2	200	0.368± 0.014	0.17 ± 0.001
3	300	0.565 ± 0.019	0.373 ± 0.001
4	400	0.776 ± 0.003	0.581 ± 0.002
5	500	1.097±1.017	0.690±0.002

***mean of three readings ± SEM**

Fig 26: Reducing power assay of ascorbic acid and ethanolic Extract of *Cordia obliqua*



From the table, it can been seen that the ethanolic extract of *Cordia obliqua leaves* showed a percentage inhibition of 0.690 ± 0.002 while ascorbic acid showed a percentage inhibition of 1.097 ± 1.01 at a concentration of 500µg/ml.

SECTION - B

ANTI-INFLAMMATORY ACTIVITY OF ETHANOLIC EXTRACT OF *CORDIA OBLIQUA* AGAINST CARRAGEENAN INDUCED PAW EDEMA IN RATS

Inflammation is a complex biological response of vascular tissue to harmful stimuli caused by injury, infection, environmental agents, malignancy and cellular changes. It is a protective attempt by the body to remove the injurious stimuli as well as to initiate the healing process for the tissue [119].

The inflammation response is a complex process that includes activation of white blood cells, the release of immune system chemicals such as complements and cytokines and the production and release of inflammation mediators and prostaglandins [120].

The complete process of anti - inflammation generally consists of three phases

1. Dilatation and increased permeability of small blood vessels resulting in oedema and swelling.
2. Emigration of leucocytes from venules and capillaries, cellular filtration and a general mopping up reaction and
3. Proliferation of fibroblast and synthesis of new connective tissue to repair the injury[121].

Similarly, the paw oedema induced by histamine, 5- HT, bradykinin, dextran, hyaluronidase and prostaglandins E have been used for studying the antagonism to those mediators.

The anti-inflammatory activities of ethanolic extract of *Cordia obliqua* at a dose of 200 mg/kg and 400mg/kg body weight were evaluated using carrageenan-induced paw oedema method. The inflammation was readily produced in the form of oedema with the help

of irritant such as carrageenan. Carrageenan is a sulphated polysaccharide obtained from sea weed (Rhodophyceae) and when injected cause the release of prostaglandins, by the way it produces inflammation and edema [121].

REQUIREMENTS:

Animal : Albino rat (180-200 g)

Drugs and chemicals : Carrageenan (1%w/v), Diclofenac sodium (standard),

: Ethanolic Extract of *Cordia obliqua.* Digital plethysmometer

METHOD:

The animals were divided into 4 groups each having six animals.

PROTOCOL

Group – 1: Treated as normal control which received 10ml/kg of normal saline through orally.

Group – 2: Treated as Standard control which received 10mg/kg of diclofenac sodium through Intraperitonealy.

Group – 3: Treated as treatment control which received 200mg/kg EECO dissolved in 2ml sterile water and administered orally.

Group - 4: Treated as treatment control which received 400mg/kg EECO dissolved in 2ml sterile water and administered through orally.

A freshly prepared suspension of carrageenan (1% w/v , 0.1 ml) was injected to the planter region of left hind paw of each rat. One group was kept as control and the animals of the other groups were pretreated with the synthetic compounds given through intraperitoneally 30 min before the carrageenan treatment. The paw volumes of the treatment

controls, standard and control groups were measured at 60,240,360 minutes of carrageenan treatment with the help of Digital plethysmometer (Ugobasile, Italy). Mean increase in paw volume was measured and the percentage of inhibition was calculated [122].

% Anti-inflammatory activity = (Vc-Vt / Vc) x 100

Where, ***Vt***- mean increase in paw volume in rats treated with test compounds,

Vc- mean increase in paw volume in control group of rats.

STATISTICS:

Data are expressed as mean ± SEM; data analyzed by one way ANOVA followed by Newman's keul's multiple range tests to determine the significance of the difference between the control group and rats treated with the test compounds.

* Values were considered significant at P< 0.01.

TABLE No.23:Anti-inflammatory activity of *EECO* by induced paw edema method

Treatment	**Dose (mg/kg)**	**Paw volume(ml) as measured by mercury displacement at 6 hour**	**Percentage inhibition of paw edema**
Group I Normal saline	10ml/kg orally	5.68±0.95	-
Group II Std	10mg/kg,i.p. Diclofenac sodium	1.85±0.35	67.42%*a
Group III	200mg/kg EECO	2.15±0.55	62.14%*a
Group IV	400mg/kg EECO.	1.90±0.66	66.54%*a

*** Data are expressed as Mean ± S.E.M.**

*Data were analyzed by one way ANOVA followed by Newman's keul's multiple range tests, to determine the significance of the difference between the control group and rats treated with the test compounds.

*a Values were significantly different from normal control at P< 0.01.

Anti- inflammatory activity

Ethanolic extract of *Cordia obliqua* at a dose of 200 and 400mg/kg were tested for their Anti- inflammatory activity by using carrageenan Induced rat paw edema method and the results were tabulated in table no 1. The results reveals that both extracts at a dose of 200 and 400mg/kg doses possesses significant Anti- inflammatory activity when compared to control group $p<0.01$.

SECTION - C

ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACT OF *CORDIA OBLIQUA WILLD.* LEAVES

Antimicrobial activity of plants can be detected by observing the growth response of various microorganisms to those plant extracts, which are placed in contact with them [123].

In order to detect antimicrobial activity in plant extracts, three conditions must be fulfilled.

First, the plant extract must be brought into contact with the cell wall of the microorganisms that have been selected for the test. Second, conditions must be adjusted so that the microorganisms are able to grow when no antimicrobial agents are present. Third, there must be some means of judging the amount of growth if any made by the test organism during the period of time choosen for the test. The currently available methods for

antimicrobial activity fall into three groups viz. diffusion, dilution and bioautographic methods [(124)].

The ethanolic extract of *Cordia obliqua* was screened for antibacterial activity on four bacterial strains and the zone of inhibition was determined.

Preparation of extract

The ethanolic extract of *Cordia obliqua* was dissolved in DMSO solution and to produce a stock solution of 2mg/ml. A quantity of 200, 400 and 600 mcg were impregnated on the plain sterile disc and dried.

Preparation of MH agar medium

Muller Hinton agar (MHmedia) was used for culture of bacterial strains. It consists of Beef 2g, casein acid hydrolysate 17.5g, starch 1.5g and agar 17g (pH 7.4 ± 0.2). MH agar (38g) was weighed and dissolved in 1000ml of distilled water and adjusted to pH 7.3 ± 0.2, sterilized by autoclaving at 121^{o}C for 15min at 15psi pressure and was used for sensitivity tests. This medium was used for screening *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* [(125)].

Preparation of Blood agar medium

Blood agar medium (BAM) contains mammalian blood usually at a concentration of 5–10%. Blood agar medium are enriched, differential media used to isolate fussy organisms and detect hemolytic activity. β-hemolytic activity will show lysis and complete digestion of red blood cell contents surrounding colony.

The media contains nutrient substrate (heart extract and peptones) 20.0; sodium chloride 5.0: agar-agar 15.0. 40g of the above media was suspended in 1000ml of distilled

water and autoclaved for 15min at 121°C and cooled to 45-50°C. The prepared medium was clear and yellowish-brown and then 5-8% defibrinated blood was added [126].

The blood agar medium was used for the antibacterial activity against the bacterial strains namely *Streptococcus pyogens*.

Preparation of bacterial cultures

The various bacterial strains like *E. coli, P.aeruginosa,* etc. were utilized for screening antibacterial activity. A few colonies of the bacterial strains picked from the agar slopes and were inoculated into 4ml peptone water in a test tube. They were incubated for 24h to form suspensions. The suspension was diluted with saline if necessary.

The visual density equivalent to standard prepared by adding 0.5ml of 1% barium chloride to 99.5ml of 1%sulphuric acid. These suspensions were then used for seeding [127].

Disc diffusion technique

The MH media was poured aseptically into sterilized petridishes and the petridishes were swirled to settle the agar and allowed to cool. The bacterial strains were seeded on the MH agar media by streaking the plate with a sterile swab containing the strain. The plain sterile discs were impregnated with various volumes of the extract, volatile oil and DMSO (negative control) and dried. Imipenum (30µg/disc) was used as standard. The discs were then placed on the plate with the help of sterile needle and the plates were incubated at 37°C for 24h [128].

Similarly the blood agar medium was used for antibacterial activity against the *Streptococcus* species and the above procedure was adopted.

The results were read and the zone of inhibition was then measured and were presented in **Tables 24**The photographic representations of the antibacterial activity were presented in **Fig 23-26& 27.**

Table No.31: Antibacterial activity of ethanolic extract of *Cordia obliqua willd* leaves.

S.No	Microorganism	Imipenum		Ethanolic extract of *cordia obliqua*	
		Concentration (µg)	Zone of inhibition(mm)	Concentration (µg)	Zone of inhibition(mm)
01	Escherichia coli	30	26	200 400 600	- 12 18
02	Pseudomonas aeruginosa		25	200 400 600	- 10 18
03	Staphylococcus aureus		24	200 400 600	- 12 18
04	Streptococcus pyogens		26	200 400 600	- 10 18

Fig.27 : Zone of inhibition of EECO against various organisms

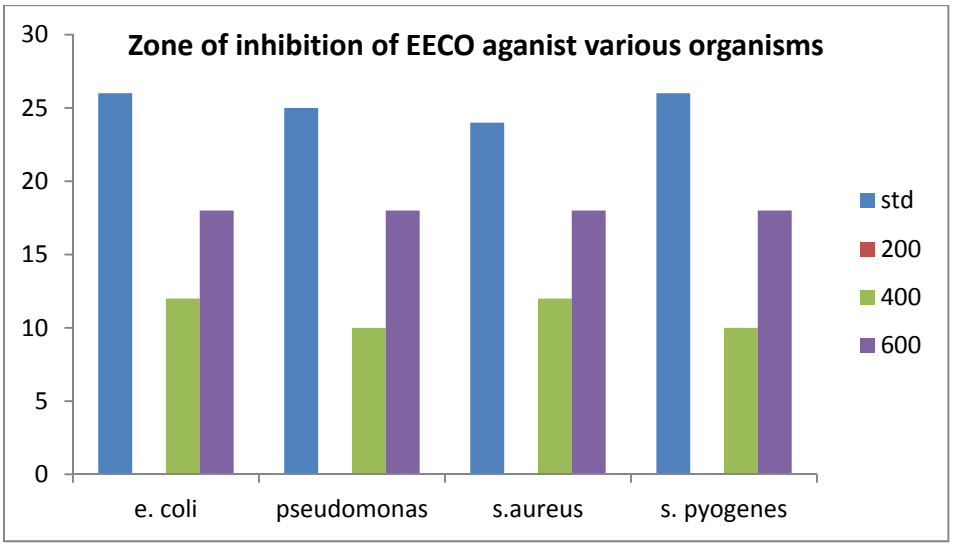


Fig. 27: Effect of *EECO* against escherichia coli

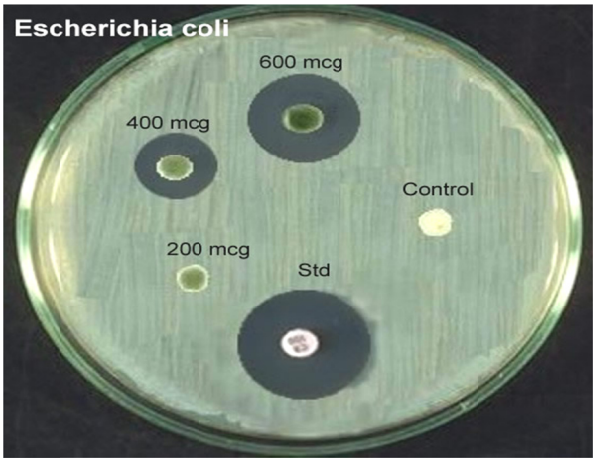


Fig.28: Effect of *EECO* against pseudomonas aeurginosa

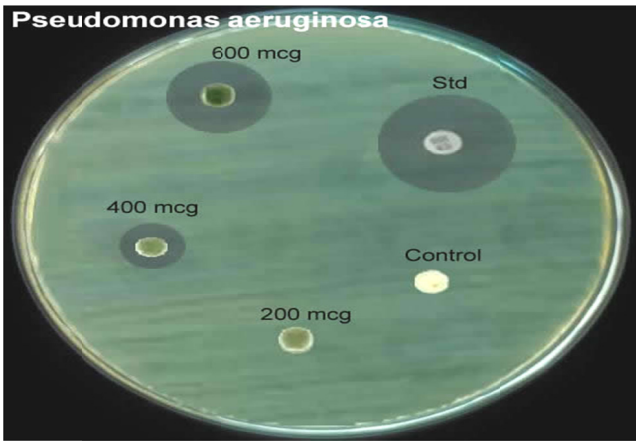


Fig. 29:Effect of *EECO* against streptococcus pyogenes

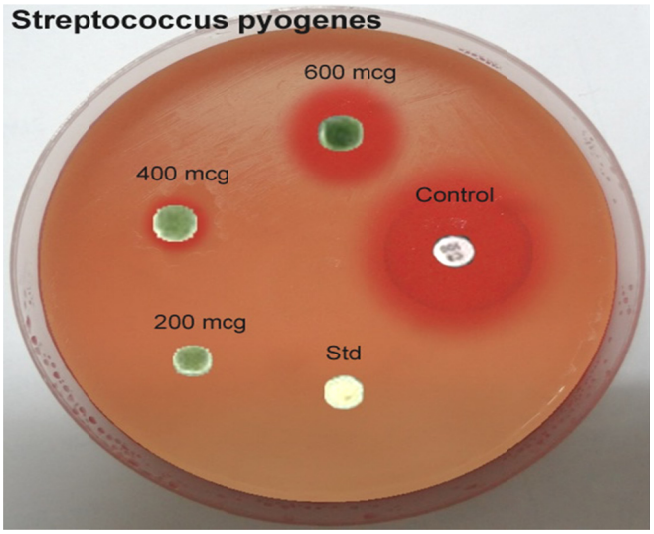
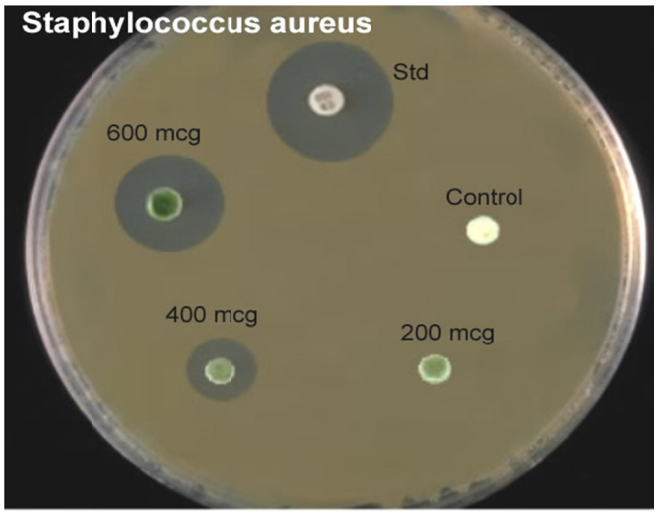


Fig.30 Effect of *EECO* against staphylococcus aureus



RESULTS AND DISCUSSION

CHAPTER – VII

RESULTS AND DISCUSSION

This dissertation deals with the Pharmacognostical, Phytochemical and Pharmacological studies of leaves of *Cordia obliqua willd.,* Family -Boraginaceae in an attempt to rationalize its use as a drug of therapeutic importance.

CHAPTER- I

This chapter gives relevant information about the traditional system of medicine, herbal medicines and the role of herbal medicines in traditional healing.Natural products for modern medicines and safety in herbal drugs were discussed in this chapter. Significance of medicinal plants to human beingswere also discussed in this chapter. Cause of inflammation, comparison between acute and chronic inflammation, chemical mediators of the inflammatory response and anti - inflammatory herbs and species were discussed in thechapter. Antibacterial activities,classification of various types of infections such as bacterial infection, mechanism of action of antibacterial agents were also discussed in this chapter.

CHAPTER- II

In this chapter, literature survey pertaining to the pharmacognostic, phytochemical, pharmacological studies and ethnomedical information of *Cordia obliqua willd* and its related species has dealt.

CHAPTER-III

Aim and scope of the study have been discussed in this chapter. Ethnomedical uses were also discussed. Pharmacognostical, Phytochemical studies which were planned to carry

out for this plant were discussed. Pharmacological studies which were planned to carry out for this plant were also discussed in this chapter.

CHAPTER-IV

In this chapter an attempt has been made to fix certain Pharmacognostic standards for the leaves of *Cordia obliqua willd* which includes the macroscopical, microscopical characters and physical parameters.

SECTION- A

Botanical name, synonyms, common name, vernacular names, taxonomic classification, geographical distribution(Habitat), description of tree, leaves, flowers, fruits and bark of the plant were described in detail, to identify the plant with the support of photographs as an establishment of authenticity.**(Fig -5-8)**

Salient features of the macroscopy of the leaves were observed, such as the leaves are alternate, ovate, 10.1 cm long, 5.7 cm broad, entire to slightly dentate margin with pinnately - reticulate venation.

SECTION- B

It deals with the microscopical studies of the leaves to ascertain the arrangement of tissues. **(Fig-9-14)**

- The leaf is dorsiventral, xeromorphic and hypostomatic.
- The midrib is thick and wide and Plano - convex in sectional view; the adaxial side is flat and the abaxial part is semicircular.
- The structure of the vascular system is complex. It consists of abaxial wide, shallow bowl shaped vascular strand and two smaller adaxial vascular strands.

- The abaxial vascular strand consists of several solitary diffusely distributed xylem elements and thick walled, lignified fibres.
- The adaxial vascular strands are collateral having a few rows of xylem elements and phloem on the outer part of the xylem.
- The adaxial epidermis is fairly thick walled; the cells are cylindrical and have prominent cuticle.
- The abaxial epidermis has smaller, thin walled cells.
- The epidermis is stomatiferous.
- The palisade cells are thin, elongated, compact and chlorophyllous.
- Calcium carbonate crystals are located in specially modified epidermal cells of the leaf.
- The epidermal cells are lightly dilated into circular (spherical) cells called lithocysts; within the lithocysts occur cystolith with a short stalk attached on the epidermal cell wall.
- Calcium oxalate crystals are large, granular bodies located in the ground parenchyma of the midrib and mesophyll cells.
- The vein- islets are narrow, squarish or polyhedral in outline.
- The vein boundary is thick. The vein -termination are short and thick. They are simple, forked once or twice.
- The trichome is nonglandular type.
- Stomata occur on the abaxial epidermis only.
- The stomata are actinocytic having three or four radiating subsidiary cells.
- The adaxial epidermis is apostomatic.

SECTION – C

It deals with the quantitative microscopy such as stomatal number, stomatal index, vein - islet number, vein termination number which were determined and tabulated as follows.

Contents	**Leaves**		
	Min	Ave	Max
Stomatal Number	53	62.5	79
Stomatal Index	45.73	48.41	58.94
Vein islet Number	9	16.9	27
Vein termination number	19	28.9	39

SECTION - D

The following physical parameters like ash values, extractive values, loss on drying, foaming index, swelling index were determined and tabulated.

ASH VALUE AND LOSS ON DRYING

Contents	**Total ash**	**Acid insoluble ash**	**Water soluble ash**	**Sulphated ash**	**Loss on drying**
Minimum	**8.86**	**0.84**	**0.94**	**0.90**	**9.32**
Average	**10.64**	**0.88**	**0.94**	**0.92**	**11.23**
Maximum	**12**	**0.90**	**0.90**	**0.94**	**10.10**
Foaming index			**<100**		
Swelling index			**3.66±0.81**		

EXTRACTIVE VALUE BY COLD MACERATION

S.No	Solvents Used (Increasing order of polarity)	Extractive Value% w/w
1	Petroleum ether	16.79
2	Ethyl acetate	1.19
3	Chloroform	2.95
4	Ethanol	2.67
5	Acetone	1.59
6	Water	18.22

.

SECTION - E

i). Fluorescence analysis of extract of *Cordia obliqua* willd

Extracts	Colour in Day light	Colour under UV Lamp 254nm	Colour under UV Lamp 365nm	Colour in visible
Acetone	Dark green	Dark green	Orange	Dark green
Ethanol	Green	Green	Orange	Light green
Ethyl acetate	Dark green	Green	Orange	Light green
Chloroform	Green	Green	Orange	Puff colour
Petroleum ether	Brown	Light green	Brown	Brown
Water	Brown	Green	Honey colour	Brown

ii). Powder analysis

The following anatomical characters were observed in the powder microscopy of leaves reveals the presence of

- Stomata - Actinocytic stomata
- Trichome – Non glandular type, bulbous basal part
- Calcium oxalate crystels
- Starch grains
- Lignified sclerenchyma
- Paranchyma

CHAPTER- VI

PHYTOCHEMICAL STUDIES

SECTION - A

This section dealt with organoleptic evaluation of the leaves of *Cordia obliqua willd.*

SECTION - B

Preliminary Phytochemical screening

This section was dealt with the phytochemical studies of the leaves of *Cordia obliqua*. This study was carried out for both powdered crude drug and various extract of *Cordia obliqua*. It reveals the presence of Carbohydrates, alkaloids, aminoacids, proteins, sterols, saponins, tannins, flavonoids and mucilage.

Constituents Present	Constituents Absent
Carbohydrates, amino acids, proteins, saponins, sterols, alkaloids, flavonoids, tannins, mucilage.	Glycosides, gums, volatile oils.

Preliminary phytochemical screening was carried out with various extracts (such as ethanol, aqueous, petroleum ether, methanol) of the leaf of this plant and the results were tabulated. **(Table. 11** and **12)**

SECTION –C

Determination of phenolic content

The total phenolic content for the ethanolic extract of *Cordia obliqua leaves* was found and shown in **Table13**. The amount of phenolic content present in the extract in terms of mg GAE/g of extract was found to be 59.47 ±1.02 by using the linear regression equation.

SECTION – D

Determination of flavonoid content

The Total flavonoid content for the ethanolic extract of *Cordia obliqua* leaves was found and shown in **Table14**. The amount of flavonoid content present in the ethanolic extract of *Cordia obliqua* leaves in terms of mg quercetin equivalent/g of extract was found to be 153.94 ± 1.20 mg/g, by using the linear regression equation.

SECTION – E

Determination of tannin content

The total tannin content for the ethanolic extract of *Cordia obliqua leaves* was found and shown in **Table 15.** The amount of tannin content present in the ethanolic extract of *Cordia obliqua leaves* in terms of mg tannic acid equivalent/g of extract was found to be165.7±1.08mg/g, by using the linear regression equation.

SECTION – F

TLC studies

Thin layer chromatography studies were carried out for the ethanolic extract of *Cordia obliqua leaves* using ethyl acetate: benzene and Chloroform: Ethyl acetate as mobile phase and the reports were presented in the **(Table.16).**

TLC STUDIES OF ETHANOLIC EXTRACT OF *CORDIA OBLIQUAWILLD.*

S.NO	DETECTING AGENT	NO OFSPOTS	Rf VALUE	COLOUR
1.	Under UV light at 365 nm	I	0.35	Yellow colour
		II	0.78	Orange fluorescences
2.	Under UV light at 365nm	I	0.52	Yellow colour
		II	0.98	Orange fluorescences

The TLC of EECO leaves when viewed under UV showed two spots at Rf value of 0.35 & 0.78 at 365nm using (Ethyl acetate: Benzene) as mobile phase and two spots at the Rf value of 0.52 and 0,98 at 365nm using (Chloroform: Methanol) as mobile phase.

SECTION – G

High performance thin layer chromatography

An attempt has been made to develop the HPTLC chromatogram for the ethanolic extract of the leaves of *Cordia obliqua,* using the mobile phase (Ethyl acetate: Toluene). The HPTLC chromatogram of ethanolic leaf extract showed twelve peaks at 254 nm and the Rf values were found to be 0.10, 0.16, 0.20, 0.32, 0.34, 0.43, 0.48, 0.58, 0.61, 0.79, 0.82, 0.91 and eleven peaks at 366nm and the Rf values were found to be 0.10, 0.16, 0.22, 0.32, 0.34, 0.43, 0.48, 0.58, 0.61, 0.79, 0.82, 0.91. (Table.17)

CHAPTER – VII

SECTION – A

Anti-oxidant activity

This part of the study dealt with the pharmacological in- vitro screening of anti-oxidant activity of *Cordia obliqua* willd., leaves by the following five methods.

PART - 1

DPPH METHOD

From the results obtained, it has been observed that the ethanolic extract of *Cordia obliqua leaves* showed a percentage inhibition of 80.16±1.44,while ascorbic acid showed a percentage inhibition of95.39± 1.28 at a concentration of 80µg/ml. The IC_{50} value, calculated by using the linear regression analysis was found to be 47.23 and 40.42µg/ml for the ethanolic extract and ascorbic acid respectively. The extract possessed a good radical scavenging activity **(Table. 18) and Fig 22**.

PART – 2

FREE RADIAL SCAVENGING ACTIVITY

From the table, it can be seen that the ethanolic extract of *Cordia obliqua* leaves showed an absorbance of 2.374 ±0.231 for a concentration of 250µg/ml, while ascorbic acid

showed an absorbance of 4.075 ±0.06 at a concentration of 250µg/ml. The extract shows a dose dependent reducing ability. The graphical representations of the reducing power activity of the ethanolic extract of *Cordia obliqua* and ascorbic acid were presented in **(Table. 19) Fig 23**

PART – 3

PHOSPHOMOLYBDENUM METHOD

From the table, it can be seen that the ethanolic extract of *Cordia obliqua* showed an absorbance of 0.261± 0.01 for a concentration of 100µg/ml, while ascorbic acid showed an absorbance of 1.1±0.02 at a concentration of 100µg/ml. The extract shows a dose dependent reducing ability. The graphical representations of the reducing power activity of the ethanolic extract of *Cordia obliqua leaves* and ascorbic acid were presented in **(Table.20)** and **Fig24.**

PART – 4

NITRIC OXIDE SCAVENGING METHOD

From the table, it can been seen that the ethanolic extract of *Cordia obliqua* showed a percentage inhibition of 138.19 ± 1.18,while ascorbic acid showed a percentage inhibition of 199.67 ± 1.10 at a concentration of 333.33µg/ml. The IC_{50} value calculated using the linear regression analysis was found to be 111.47and 83.47µg/ml for the ethanolic extract and ascorbic acid respectively.

PART – 5

REDUCING POWER ASSAY

From the table, it can been seen that the ethanolic extract of *Cordia obliqua* leaves showed a percentage inhibition of 0.690 ± 0.002, while ascorbic acid showed a percentage inhibition of 1.097± 1.01.The graphical representations of the reducing power activity of the

ethanolic extract of *Cordia obliqua* and ascorbic acid were presented in **Fig 26** showed a percentage inhibition of 1.097 ± 1.01 at a concentration of 500µg/ml **(Table 22).**

SECTION – B

Anti - inflammatory activity

This study deals with the In-vivo pharmacological screening of the ethanolic extract of *Cordia obliqua* leaves at a dose of 200 and 400mg/kg, for their anti- inflammatory activity by using carrageenan Induced rat paw edema method and the results were tabulated in **Table 23**. The results reveals that both the extracts at a dose of 200 and 400mg/kg, possesses significant Anti- inflammatory activity when compared to control group ($p<0.01$).

SECTION – C

Antibacterial activity

This study deals with the In-vitro pharmacological screening of ethanolic extract of Cordia obliqua leaves for the antibacterial activity by the disc diffusion technique. The results obtained indicate that the ethanolic extract of *Cordia obliqua* leaves had significant effect on all the microorganisms tested. EECO was tested for the antibacterial activity against *Escherichia coli, Pseudomonas aurogenosa, Staphylococcus aureus and streptococcus pyogens.* Zone of inhibition of EECO was found to be, for ***Escherichia coli*** *400µg -12mm, 600µg-18mm,* ***Pseudomonas aurogenosa*** *-400 µg-10mm, 600µg -18mm ,* ***Staphylococcus aureus*** *-400µg -12mm , 600µg -18mm,* ***Streptococcus pyogenes*** 400µg -10mm , 600µg-18mm) compared to imipenum. These results indicate that EECO has exerted signigicant antibacterial activity against all the four bacterial pathogens at 400µg and 600 µg/ml concentrations, which is comparable with 30µg imipenum **(Table 24)** and **Fig. 27- 31.**.

CONCLUSION

CHAPTER- VIII

CONCLUSION

This dissertation covers the pharmacognostical parameters of the leaves of ***cordia obliqua willd.,*** belonging to the family **Boraginaceae**. The macroscopical , microscopical characters, powder analysis, quantitative microscopy and physical standards like ash values, extractive values, loss on drying, swelling index, foaming index, fluorescence analysis have been studied and presented.

The gist of the findings derived from the investigations under this project is furnished in the following concluding lines.

Pharmacognostical parameters have been determined for the leaf of *Cordia obliqua i*n order to substantiate and identity the plant for future work.

The kinds of microstructure have been recognized as tools to measure the phylogenetic relationships under light microscope, to resolve taxonomic controversies and to establish the botanical identity of the plant.

Preliminary phytochemical screening of the leaf of *Cordia obliqu*a reveals the presence of sterols, carbohydrates, aminoacids, proteins, alkaloids, saponin, flavonoids, tannins and mucilage.

Determination of phenolic content, flavonoid and tannin content confirms that significant concentrations of these phyto constituents are present in the ethanolic extract of leaves of *cordia obliqua willd.*

TLC studies were performed with the ethanolic extract of the leaf to identify the phytoconstituents present in the leaf of this plant.

HPTLC finger print studies were performed to evaluate the active constituents present in the leaf of the plant.

Pharmacological screening confirms that the ethanolic extract of *Cordia obliqua* leaves have showed significant antioxidant activity which was evaluated by

Free radical scavenging by DPPH assay

Ferric Reducing Power Assay

Nitric oxide scavenging assay

Phosphomolybdenum Assay

Reducing power Assay

Ethanolic extract of Cordia obliqua leaves also showed significant anti- inflammatory activity which was evaluated by

Carrageenan induced paw edema method.

The ethanolic extract of *Cordia obliqua* leaves (EECO) exhibited antibacterial activity against *Escherichia coli, pseudomonas aeruginosa, staphylococcus aureus and streptococcus pyogens.* Anti- bacterial activity of this extract against these microorganisms was significant comparable with standard drug imipenum.

The above results reveal that, the ethanolic extract of *Cordia oblique* leaves (EECO) had showed significant antioxidant anti- inflammatory activity and moderate antibacterial activity. These activities may be due to the presence of the phytoconstituents like flavonoids, tannins etc., present in the leaves of *Cordia obliqua.*

Future aim of this study is to isolate the pharmacologically potent phytochemicals responsible for the above activities and detailed investigation for its exact mechanism of action which can result in complimentary to those of existing anti-inflammatory, antibacterial and antioxidant agents to deduce a definite Conclusion.

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Thank
You